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ASYN and Tau Interaction: New Drug Target for Neurodegenerative Diseases

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ASYN and Tau Interaction: New Drug Target for Neurodegenerative Diseases

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Tese co-orientada pela Doutora Patricia Calado e pela Prof. Doutora Margarida D. Amaral, especialmente elaborada para a obtenção do grau de doutor em Bioquímica, especialidade de Biotecnologia

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(To my family, for always supporting me and being present)

A Silvia
"S'agapò tora ke tha s'agapò pantote"
Oriana e Alekos

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Abstract

Neurodegenerative diseases are among the most complex and puzzling human disorders and in the last century the number of people affected by neurodegenerative disorders is increasing year after year. These devastating disorders currently do not have any effective therapies or treatments, thus are a social and economic burden for modern society and novel therapeutic strategies need to be developed for these disease states.

Synucleinopathies and tauopathies regroup a wide number of neurodegenerative disorders characterized by the presence of abnormal protein aggregates respectively composed of alpha-synuclein (ASYN) or tau protein. Several reports revealed a consisting overlapping between these two groups of disorders and that the interactions between ASYN and tau may be a relevant disease component that enhances the pathological cascade and spreads the neuronal damage. Budding yeast have been largely used to perform studies on the physiopathology of synucleopathies and tauopathies and are a powerful tool for rapid screening assays that have resulted in the identification of several promising therapeutic drugs and targets.

In this work we used yeast as a model system to reproduce the synergistic cytotoxic effect mediated by the co-expression of the human ASYN and tau proteins that recapitulates some of the pathological features observed in patient's brain. The model was used to perform genome-wide screening and high-throughput assays to identify both genes and natural extracts able to modulate the synergistic cytotoxic interactions of ASYN and tau.

In the end, we were able to identify 5 different *S. cerevisiae* genomic fragments containing a total of 25 different complete genes and 11 natural extracts able to interfere with the reported synergistic cytotoxicity. These modulators have the potential to be further explored in other appropriate disease models and might be relevant for academic groups and companies working on neurodegeneration engaged in drug discovery.

Keywords: Neurodegeneration, alpha-synuclein, tau, yeast, screening.

Resumo

As doenças neurodegenerativas representam uma das patologias humanas mais complexas e desafiadoras, sendo que no último século o número de pessoas afetadas tem crescido anualmente. Estas doenças devastadoras não têm atualmente nenhuma terapia ou tratamento eficaz, pelo que são um fardo social e económico para a sociedade moderna tornando urgente o desenvolvimento de novas estratégias terapêuticas para estes estados patológicos.

O termo sinucleinopatias agrupa várias doenças neurodegenerativas diferentes em que a característica patológica marcante é a presença de agregados fibrilares insolúveis da proteína alfa-sinucleína (ASYN), designados por corpos de Lewy, em populações específicas de células do cérebro. Este grupo de patologias inclui a doença de Parkinson, a demência com corpos de Lewy, a atrofia multissistémica, muitos casos de doença de Alzheimer (variante da doença de Alzheimer com corpos de Lewy), neurodegeneração com acumulação de ferro tipo I, falência autonómica pura, e um subtipo de tremor essencial. Na maioria dos casos as sinucleinopatias são doenças idiopáticas, envolvendo mutações poligénicas, interações génicas e de estilo de vida. Em 1997 o gene da alfa-sinucleína foi o primeiro gene que se descobriu estar associado à doença de Parkinson.

De igual forma, as tauopatias representam outro grupo de doenças neurodegenerativas caracterizadas pela presença de agregados neurofibrilares compostos maioritariamente pela proteína tau anormalmente hiperfosforilada. Patologias como a doença de Alzheimer, degeneração lobar frontotemporal, demência frontotemporal com parkinsonismo ligado ao cromossoma 17, paralisia supranuclear progressiva e a degeneração córtico-basal são exemplos de tauopatias. O estado de fosforilação da proteína tau tem um papel central na etiologia das tauopatias, contudo um elevado número de mutações no gene tau foi também identificado e associado com algumas destas doenças.

As sinucleinopatias e as tauopatias partilham mecanismos moleculares comuns conducentes à neurodegeneração e os processos patogénicos através do qual as proteínas ASYN e tau induzem morte celular estão relacionados com auto

agregação levando à formação de espécies oligoméricas potencialmente citotóxicas que por sua vez conduzem a alterações em vários processos celulares, nomeadamente na sinalização celular dependente de cálcio, atividade mitocondrial, estrutura do citoesqueleto, sistema de controlo de qualidade de proteínas, propagação célula a célula e ativação da resposta imune. Mais ainda, recentemente foram descobertas muitas interações diretas e indiretas entre estas duas proteínas, sendo de salientar a identificação de toxicidade sinergística aquando da co-expressão de ASYN e tau em modelos de mamíferos e de levedura. Tendo em conta todas estas analogias na fisiopatologia das sinucleinopatias e das tauopatias e a citotoxicidade sinergística entre ASYN e tau, estas duas proteínas podem representar um duplo alvo para o desenvolvimento de novas abordagens terapêuticas para o tratamento de um vasto número de doenças neurodegenerativas.

Apesar da ausência de um sistema nervoso os modelos de levedura são amplamente utilizados para abordagens às doenças neurodegenerativas, tendo em conta que muitos dos processos envolvidos na progressão patológica, tais como atividade mitocondrial, regulação da transcrição, tráfico intracelular e controlo de qualidade de proteínas, são muito conservados entre as leveduras e os humanos. Adicionalmente, cerca de 30% dos genes humanos envolvidos no desenvolvimento de muitas doenças humanas têm ortólogos funcionais em levedura. As células de levedura são também usadas para o rastreio em larga escala de bibliotecas de compostos devido a vantagens como a avaliação de compostos num ambiente fisiologicamente relevante e a seleção negativa imediata de compostos tóxicos ou com baixa permeabilidade membranar. Mais ainda, estão disponíveis coleções de estirpes de levedura mutadas por deleção, juntamente com coleções de sobre-expressão que constituem ferramentas poderosas para ensaios de análise genómica - *genome-wide screening*.

No trabalho descrito nesta tese utilizámos a levedura como sistema modelo para reproduzir e caracterizar o efeito citotóxico sinergístico mediado pela co-expressão das proteínas ASYN e tau humanas. O modelo foi depois usado como uma ferramenta para a descoberta de genes e compostos que medeiam a interação

tóxica entre ASYN e tau, possibilitando a identificação de novos alvos para intervenção terapêutica.

Começámos por promover a expressão de ASYN e tau em levedura a partir de um vetor episssomal bi-direcional. Os resultados obtidos mostraram que a presença de ASYN afeta a solubilidade da proteína tau, aumentando a fração de proteína insolúvel/agregada em associação com um aumento significativo na fosforilação de tau nos epítopos patogénicos S396/404, o que se sabe levar à formação de agregados de tau insolúveis. Mais ainda, mostrámos que no nosso modelo a presença de ASYN conduz ao aumento de agregação de tau no epítopo S396/404 via Rim11, o ortólogo de levedura de GSK3B. Estas descobertas estão alinhadas com outros relatos em que se provou que o epítopo patológico S396/404 é um substrato típico de GSK3B e que a ASYN tem a capacidade de estimular diretamente a fosforilação de tau via GSK3B, fazendo parte de um complexo heterotrimérico contendo ASYN, tau e GSK3B. Apesar do aumento na fosforilação e agregação de tau promovido pela co-expressão de ASYN, não foram observadas diferenças sinergísticas no fenótipo de crescimento do nosso modelo episssomal de levedura. Contudo, esta foi a primeira evidência que a ASYN tem capacidade de induzir a fosforilação e agregação de tau em levedura.

Avaliámos de seguida o efeito da co-expressão de ASYN e tau em levedura recorrendo a uma abordagem experimental diferente, baseada na integração estável de uma cópia de cada transgene no genoma da levedura. Neste cenário, foi observado um forte efeito citotóxico sinergístico no crescimento da levedura, associado com um aumento da fosforilação de tau no epítopo S396/404 e a um aumento da fração insolúvel de proteína tau. De salientar que esta citotoxicidade sinergística é eliminada aquando da remoção de *RIM11*, demonstrando que o fenótipo obtido é resultante da cooperação entre ASYN e tau. Estes resultados sugerem que a hiperfosforilação de tau no epítopo S396/404 mediada por ASYN pode dar origem a espécies oligoméricas citotóxicas de tau, uma vez que esta citotoxicidade sinergística só é observada na estirpe de levedura que apresenta níveis mais elevados de tau fosforilada, em correlação com a presença de tau na fração proteica insolúvel.

Em resumo, as nossas estirpes de levedura, episssomal e integrativa, recapitulam muitos dos aspetos mais relevantes das interações de ASYN e tau na patologia humana e são ferramentas poderosas para ensaios de rastreio rápidos. Em particular, a estirpe integrativa, que revela um fenótipo sinergístico tóxico devido à co-expressão de ASYN e tau, foi usada para a realização de um *genome-wide screening* (GWS) e de um *high-throughput screening* (HTS) com o objetivo de identificarmos genes e compostos que possam futuramente ser alvos para o desenvolvimento de novas terapias para doenças neurodegenerativas.

O GWS foi realizado usando a colecção "Yeast Genomic Tiling Collection Assay Ready DNA", que consiste em cerca de 1500 clones, cada um contendo um segmento único do genoma da levedura *S. cerevisiae* num vetor shuttle (*E. coli* – levedura) e fornecida como uma *pool* de DNA num tubo único. O GWS resultou na identificação de 5 fragmentos genómicos diferentes com um total de 25 genes completos que, quando sobre-expressos, têm a capacidade de diminuir fortemente a citotoxicidade sinergística mediada por ASYN e tau. Apesar dos nossos esforços iniciais para identificar os genes responsáveis por esta recuperação de fenótipo não terem sido bem sucedidos, a análise destes fragmentos genómicos deve ser alvo de atenção futura com vista a compreender quais os genes individuais ou interação de genes que medeia este efeito benéfico.

O HTS foi realizado utilizando a coleção proprietária LUSOEXTRACT, que consiste em 3932 extratos naturais provenientes de 1206 organismos isolados de ecossistemas aquáticos e terrestres Portugueses únicos. A análise final do HTS resultou em 11 extratos naturais com capacidade para melhorar o efeito citotóxico mediado pela expressão concomitante de ASYN e tau, sendo que estes extratos devem ser alvo de estudos posteriores neste e noutros modelos celulares de doença.

A identificação de moduladores da toxicidade sinergística mediada por ASYN e tau é relevante tanto para grupos académicos que trabalham em neurodegeneração como para empresas que desenvolvem programas de identificação de novos fármacos. Temos a esperança que os resultados do presente trabalho possam no futuro ter um impacto positivo nos doentes, famílias

e prestadores de cuidados de saúde que lidam diariamente com a falta de alternativas terapêuticas para as doenças neurodegenerativas.

Palavras-chave: Neurodegeneração, alfa-sinucleína, tau, leveduras, screening.

Abbreviations

AD	Alzheimer’s disease
AJPR	autosomal recessive juvenile parkinsonism
ALP	autophagosome-lysosome pathways
ALS	amyotrophic lateral sclerosis
AP-1	jun proto-oncogene
ASYN	alpha-synuclein protein
ATP	adenosine triphosphate
AVs	autophagic vacuoles
Aβ	amyloid beta
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CDK5	cyclin-dependent kinase 5
CMA	chaperone-mediated autophagy
CNA	central nervous system
DAMPs	danger-associated molecular patterns
DLB	dementia with Lewy bodies
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated degradation
FTD	frontotemporal dementia
FTDP-17	frontotemporal dementia with parkinsonism linked to the chromosome 17q21-22
FTLD	frontotemporal lobar degeneration
GSK3B	glycogen synthase kinase 3 beta
GWAS	genome wide association studies
GWS	genome-wide screening
HD	Huntington’s disease
HNE	4-hydroxy-2-nonenal
HTS	high-throughput screening
JNK	c-jun kinases
LB	Lewy bodies
MAPKs	mitogen-activated protein kinase superfamily
MAPT	microtubule associated protein tau
MARK	microtubule affinity regulating kinase
MCI	mild cognitive impairment
MPTP	1-methyl-4-phenyl-1,2,3,6-teteahydropyridine
mRNA	messenger RNA
MSA	multiple system atrophy
MT	microtubule
MTBD	microtubule binding domain
mtDNA	mitochondria DNA

NAC	non-amyloid beta component
NBIA	neurodegeneration with brain iron accumulation
NFTs	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PAF	pure autonomic failure
PCD	programmed cell death
PD	Parkinson’s disease
PDPKs	proline-directed protein kinases
PKA	AMP-dependent protein kinase
PQC	protein quality control
RABS	ER-to-Golgi vesicular trafficking proteins
ROS	reactive oxygen species
rRNA	ribosomal RNA
SAPKs	stress-activated protein kinases
SNARE	soluble N-ethylsaleimide-sensitive fusion protein attachment protein receptor
SNCA	synuclein, alpha gene
STEP	striatal-enriched protein tyrosine phosphatase
TKs	tyrosine kinases
TLRs	Tool-like receptors
tRNA	transport RNA
UPR	unfolded protein response
UPS	ubiquitin-proteasome system

Chapter I. Introduction

1. Brain Diseases

1.1. Neurodegenerative Diseases Overview

According to the Alzheimer's Foundation of America, dementia is a general term that describes a group of symptoms—such as loss of memory, judgment, language, complex motor skills, and other intellectual functions caused by the permanent damage or death of the brain's nerve cells, or neurons. The group of disorders causing dementia includes Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS), and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). AD is the most common cause of dementia in people over 65 and represents about 60% of all dementias, whereas vascular dementia caused by stroke or blockage of blood supply accounts for about 30% [1-3]. Epidemiologic studies predict that the number of people with dementia will double each 20 years in developed and developing countries, with a rate three to four times higher in developing areas than in developed regions, as consequence of increased life expectancy and exposure to many other risk factors (Figure 1).

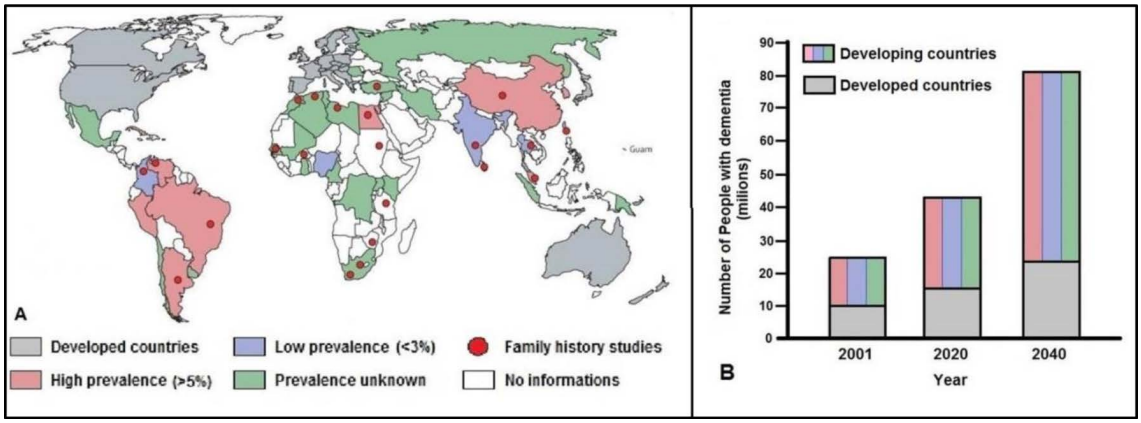

Figure 1: Worldwide dementia prevalence and projection in developed and developing countries. A) Red countries have a prevalence estimated to be higher than 5%, which is similar to the one estimated in developed countries (in grey). Blue countries have a prevalence of dementia lower than 3% while in green countries cases of dementia have been reported but

prevalence or incidence are unknown. No information is available from white countries. Red spots show locations of families with neurodegenerative disorders causing dementia [3]. **B)** The plot is representative of a study were it was estimated that 24 million people had dementia in 2001 and this amount will double every 20 years to reach 42 million by 2020 and 81 million by 2040 [2].

Aging is well known to be the greatest risk factor for this group of disorders, however the neuronal loss caused by intracellular oxidative stress accumulation in elderly peoples is much lower than the one observed in chronic neurodegenerative process [4]. All the neurodegenerative diseases are mainly characterized by an initial abnormal neuronal activity that progressively leads to neuronal cell death [5,6] due to the impairment of many intracellular processes including protein degradation and mitochondrial biology [7,8]. Another common hallmark of several neurodegenerative diseases is the presence of plaques and other atypical protein aggregates composed by normal or mutated disease-related proteins with different intra or extra cellular localization. Depending on the disease subtype, the distribution of these aggregates can be mainly cytosolic, predominantly intranuclear, in the endoplasmic reticulum (ER) or extracellular [9], but their direct contribute on the pathology progression is still under debate [9,10].

1.2. Aging Brain: The Biggest Risk Factor in Neurodegeneration

Neurodegenerative diseases share a common risk factor, the aging of the brain. In most cases aging and cognitive decline walk side by side, although the molecular mechanisms involved in aging are still unclear. Another important issue to address is how the physiological brain aging gives rise to neurodegenerative disorders. In normal elderly human population the cognitive decline—also called mild cognitive impairment (MCI)—is represented by delayed recall of verbal information, regression of working memory, short-term memory, reduced spatial memory and processing speed which is the most affected, then normal aging people take longer to learn new information [11-15]. Other cognitive functions such as long-term memory, implicit memory, attention span, vocabulary and verbal knowledge are well preserved during the aging process and emotional components of memory seem to be even improved

especially in people over 65 [16]. The memory functions compromised during aging may be the result of activation changes in the hippocampus and prefrontal cortex brain regions as revealed by functional imaging analysis [17,18]. Activation of the contralateral hemisphere was also shown in normal aging brains, representative of the normal compensatory response known as “loss of hemispheric asymmetry” that is lost in MCI and in AD [19]. AD human brains revealed an over-time accumulation of protein aggregates together with a progressive massive neuronal loss in the medial temporal lobes, in particular in the entorhinal cortex in contrast with the normal aging brains where a mild cell loss was observed only in the prefrontal cortex (Figure 2).

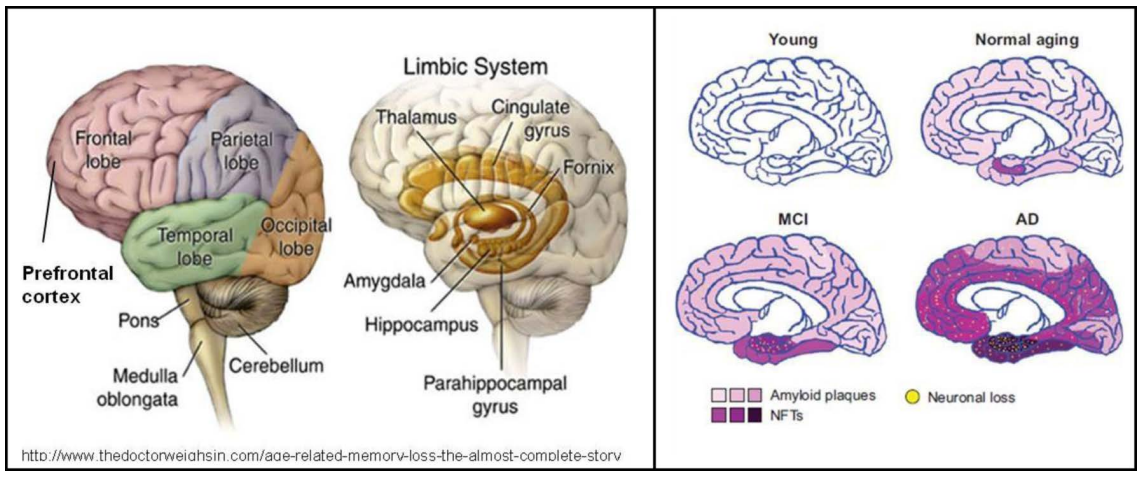

Figure 2: Brain structure and pathology progression. In MCI, hippocampus and prefrontal cortex are the affected regions marked by neuronal loss accompanied by a diffuse moderate presence of amyloid plaques composed by Aβ peptide and neurofibrillary tangles composed by tau protein. Remarkable general volume loss and selective neuronal cell death in temporal lobe in association with widespread massive presence of protein aggregates was observed only in AD human brains [4,20].

Loss of synaptic function was also observed in the frontal cortex of normal aging brain [21]. Synaptic plasticity is a calcium-dependent function and reduced mRNA levels of calcium channel subunits and key proteins in the calcium signal pathways have been demonstrated in the aging prefrontal cortex [22]. Several microarray studies on human aging brain have been performed to evaluate changes in gene expression and in the frontal cortex about 4% of the active genes are regulated during the aging process [22,23]. Genes involved in memory, learning, synaptic plasticity, vesicle protein transport and mitochondrial function were significantly down-regulated while genes involved in stress response, in

particular antioxidants, DNA repair and inflammation, were up-regulated (Figure 3). Thus, age-related molecular changes at several levels expose neuronal cells to oxidative stress and biological dysfunction, compromising the entire biological systems of the brain. In accordance, the risk of AD onset increases 14-fold from the age of 65 to 85, afflicting about 47% of people over 85 [24]. Moreover, the presence of abnormal protein aggregates composed of amyloid-β peptides (Aβ) in AD, of tau protein in FTLD and of alpha-synuclein (ASYN) protein in PD is considered a potential contributory factor [10], but the current animal models do not explain the interconnections between aging and diseases onset.

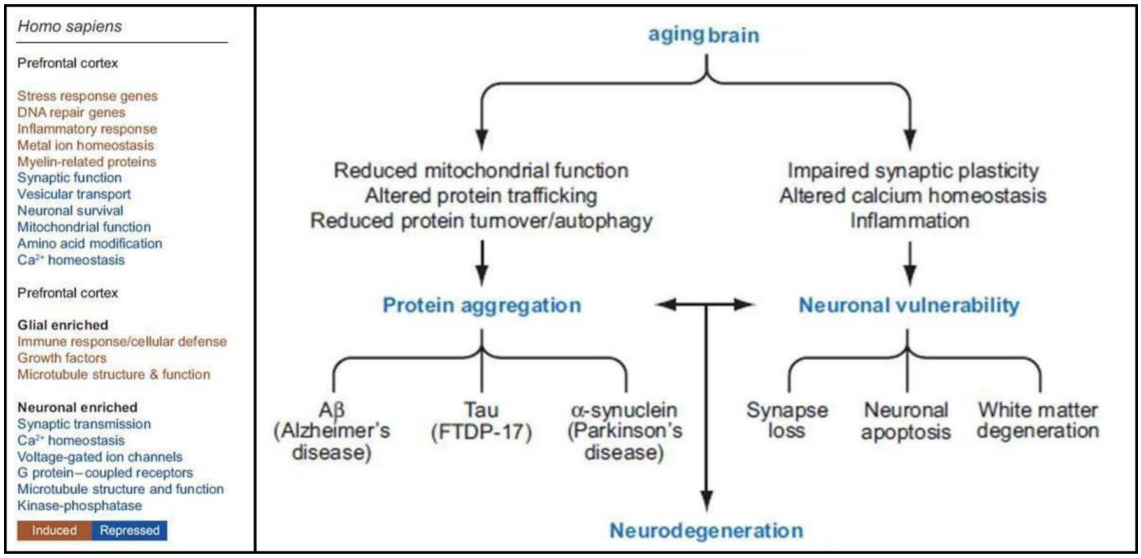

Figure 3: Aging and neurodegeneration. During the aging process changes in gene expression (left panel) might contribute to impaired intracellular pathways rendering the neurons more sensitive to several stress insults. This age-dependent neuronal vulnerability may represent the link between aging and neurodegeneration [4].

2. Neurodegeneration Mechanisms

2.1. Failure of Neural Network

The early impairment of neural activity plays a crucial role in the manifestation of neurodegenerative disorders. The evidence of neural network failure is the manifestation of impressive fluctuation in functional abilities–disabilities within the same day in people affected by neurodegenerative disorders which significantly correlate with neuropsychological and electrophysiological measures [25-27]. Functional reversible fluctuations could be the result of impaired intra–extra cellular processes not related to significant

neuronal death, such as molecular signalling, synapses activity, neuronal activity and neural network (Figure 4) [28-30]. This thesis is strongly supported by many studies in transgenic models expressing normal or mutant proteins related to AD, PD, HD or other neurodegenerative diseases. Results obtained showed how impaired behaviour and neuronal deficit observed without neuronal loss could be restored or prevented upon removal of the diseases related proteins [31-34].

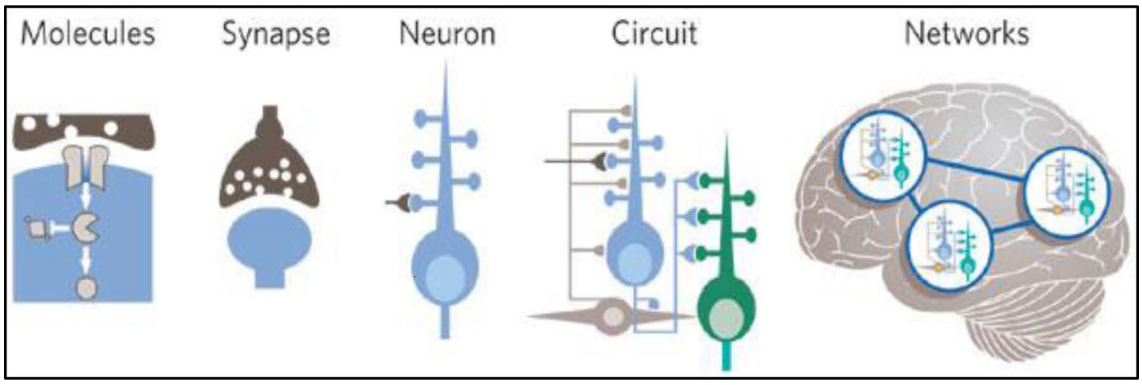

Figure 4: Neurodegenerative disorders affect neural activities at many levels. Neurodegenerative disorders can disrupt molecular pathways, synapses and local circuits in specific brain regions, as well as higher-order neural networks. Abnormal network activities may result in a vicious cycle, impairing the integrity and functions of neurons and synapses, for example, through aberrant excitation or inhibition [5].

For instance, in AD the cellular mechanisms involved in network failure are the ones linked to:

- **Activity-regulated genes** such as those encoding for cytoskeletal proteins, long-term potentiation, memory formation and immediate-early proteins forming the transcription factor AP-1 [35-39].
- **Cell membrane receptors** including cell adhesion proteins, glutamate receptors, nicotinic acetylcholine receptors and NMDA receptors [35,40-44].
- **Signalling cascades** regulated by several kinases and phosphatase like the mitogen-activated protein kinase superfamily (MAPKs), cyclin-dependent kinase 5 (CDK5), tyrosine kinases (TKs), calcineurin phosphatase and striatal-enriched protein tyrosine phosphatase (STEP) [35,40,43,45-48].
- **Synaptic integrity** controlled by presynaptic terminals and postsynaptic dendritic spines [49-51].
- **Neurotransmitter synaptic release** via vesicles cycling [52].

Dysregulation of the described pathways triggers the activation of complex compensatory mechanisms that recruit different neuron subtypes to restore the normal activity [53,54]. This compensation could explain the slow progression of most neurodegenerative disorders and why so many neurons can die before the manifestation of relevant clinical symptoms. Progressive failure of these compensatory mechanisms may contribute to the remarkable daily fluctuations.

2.2. Protein Aggregation and Degradation

The cellular environment is composed by millions of proteins packed in their respective subcellular compartments. Maintaining a proper protein homeostasis under intrinsic and environmental conditions is essential for long-term cells health and to preserve the correct biological functions. Protein homeostasis involves transcription regulation, translation, folding, trafficking, processing, assembly–disassembly, localization and degradation. Many neurodegenerative diseases are characterized by the accumulation of misfolded proteins in insoluble inclusions with different anatomical distribution depending on the disease (Figure 5) [55].

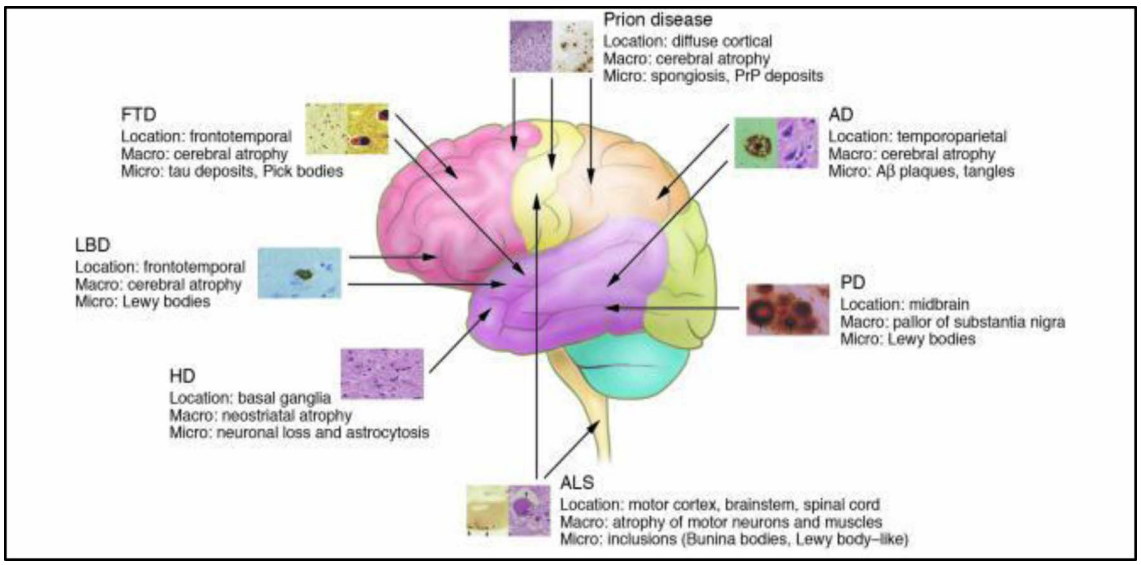

Figure 5: Anatomical location of protein aggregates. Macroscopic and microscopic changes observed on the most common neurodegenerative disorders [55].

AD and PD are respectively the first and second most common age-related neurodegenerative diseases characterized by the presence of protein aggregates. In AD the extracellular amyloid plaques are generated from the sequential

cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase, giving rise to Aβ peptides of 40 and 42 amino acids residues called $A\beta_{40}$ and $A\beta_{42}$ [56-58]. Numerous heritable mutations in the APP are linked to AD onset during the first fifth decade of life, while the majority of the sporadic cases of AD develop clinical onset in people of 70 years old or more [24]. The other major hallmark in AD is the presence of neurofibrillary tangles (NFTs) which are intracellular insoluble fibrillar deposits formed by hyperphosphorylated tau protein [59,60]. Furthermore, more than 50 different mutations in the microtubule associated protein tau (*MAPT*) gene have been associated to familial forms of frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [61,62]. PD is characterized by the presence of Lewy bodies (LB) which are intracellular cytoplasmic aggregates composed by the presynaptic ASYN protein and the cells affected are mainly the dopaminergic neurons in the substantia nigra [61]. The majority of PD cases are sporadic, however duplication and triplication of the synuclein-alpha (*SNCA*) locus gene as well point mutation in the ASYN proteins (A30P, E46K and A53T) have been identified in early-onset PD patients [63,64].

All these aggregates are composed by misfolded or unfolded disease-related proteins organized in a well-defined structure or amorphous, in both cases insoluble and refractory to proteolysis [65]. It is currently known that genetic mutations, environmental factors and different stress conditions are all able to induce protein misfolding and aggregation (Figure 6).

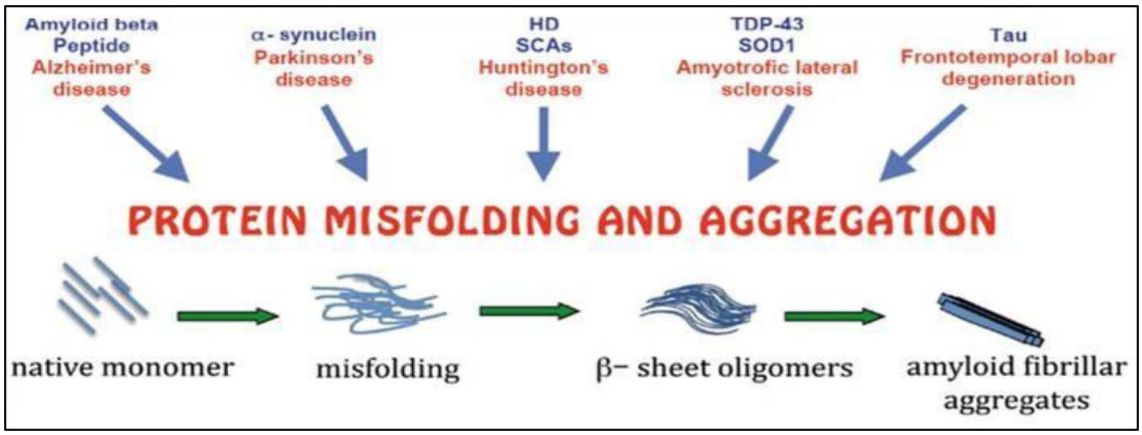

Figure 6: Misfolding and aggregation. Protein aggregation is a natural phenomenon occurring in living organisms, although in several neurodegenerative disorders the disease related proteins start to self-aggregate in oligomeric intermediates that evolve into fibrillar structures.

Misfolding and aggregation can be promoted by genetic mutations, intra–extra cellular concentration, abnormal post-translational modification, proteolyitic cleavage, aging and environmental factors [66,67].

Recent studies revealed that those protein aggregates may diffuse from one affected cell to one normal cell, acting as seeds to initiate protein misfolding and aggregation in a process knew as “prion-like diffusion” [68]. In general it seems that the aggregation ability correlates with the cytotoxicity, but the direct implication of soluble monomer, oligomers or larger protein aggregates with the pathology progression is still under debate [69-71]. Several contrasting results have been observed and produced in the last century since Alois Alzheimer in 1907 discovered for the first time the presence of amyloid plaques in post-mortem brains from demented patients [72]. Two early hypotheses have been formulated to correlate neurodegenerative diseases with proteins aggregates: **A)** human neurodegenerative disease causes fibrillar deposits, but protein aggregation has no causal role; **B)** fibrillar protein deposits cause neurodegenerative disease. Several evidence argues against both hypotheses, as it was observed that some subtypes of neurodegenerative disorders are characterized by the absence of protein aggregates, as happens in the genetic prion disorder Gerstmann–Straussler syndrome, in sporadic ALS and in autosomal recessive juvenile parkinsonism (AJPR) [73-75]. Furthermore, dissociation between the severity of the symptoms and the number and the size of amyloid deposits in post-mortem AD brains was proved [76,77], and amyloid plaques were also found in non-demented elderly peoples [78]. Contrasting results have also been produced in neurodegenerative models where, in some cases, symptomatic reversal occurs upon removal of the aggregation-prone proteins and correlate with the disappearance of intracellular tangles [79-81] while in other cases fibrillary deposits are not directly related to diseases symptoms [82-84]. Moreover, amyloid fibrils can continue to grow and multimerize to form larger aggregates that evolve into an aggresome. It has been suggested that the aggresome is a protective structure formed to regroup potential proteasome-resistant toxic aggregates that will be then eliminated by autophagy [65,85]. However, even if it is not currently possible to directly correlate the

amount of a discrete protein aggregate to a human disease, the intracellular pathways involved in the maintenance of a proper protein homeostasis are largely studied as potential therapeutic targets for the treatment of neurodegenerative disorders. The first line of defence against misfolded proteins prone to aggregation are the molecular chaperones that can stabilize or refold such protein species and are also associated with the protein quality control (PQC) system in order to promote the elimination of those proteins that can no longer be refolded [86-90]. The PQC is regulated by two main distinct pathways, the ubiquitin-proteasome system (UPS) and the autophagosome-lysosome pathways (ALP). The successive failure of these protein degradation pathways, as cause or consequence of early pathological alterations in affected neurons, might represent a key step in the pathological cascade that gives rise to spreading neurodegeneration. Within the UPS pathway the key structure is the proteasome, a barrel-shaped multiprotein complex able to hydrolyse not only cytosolic and nuclear soluble proteins but also misfolded proteins inside the ER through a process known as endoplasmic-reticulum-associated degradation (ERAD) (Figure 7) [91,92].

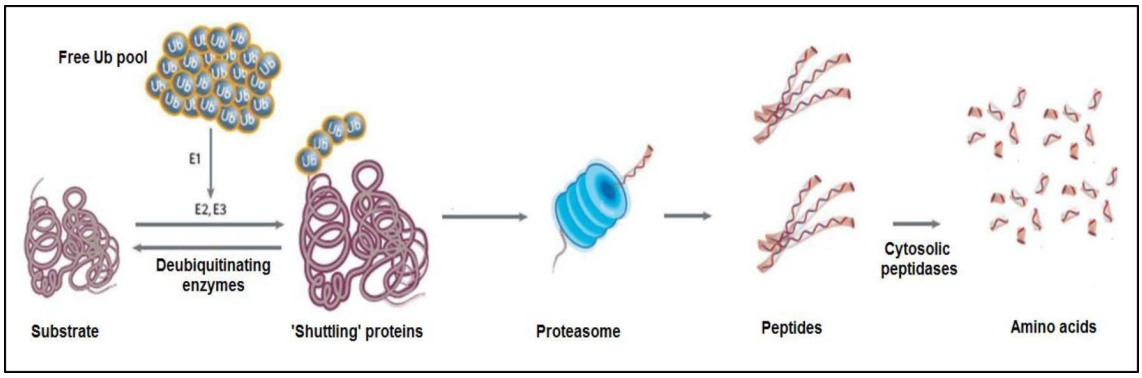

Figure 7: UPS system. Proteasomal degradation is ubiquitin (Ub) dependent. Proteins tagged with chains of four or more ubiquitins are shuttled to the proteasome by various proteins. The ubiquitin conjugation requires three different subtypes of enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase enzyme (E3). In the proteasome, proteins are hydrolysed to peptides, which are then released into the cytosol and further broken down by peptidases [93].

The target signal for protein degradation is the presence of a chain composed by four or more ubiquitin molecules covalently bound at the C-terminal region [93]. The polyubiquitinated targeted proteins need to be unfolded to pass through the

proteasome barrel pores to be hydrolysed, thereby oligomeric and aggregated proteins are resistant to UPS clearance [87]. Most of the proteins involved in neurodegeneration, such as ASYN or tau, are UPS dependent for their clearance before they start to self-aggregate [9,91]. When a cytosolic aggregation-prone protein becomes a poor proteasome target the ALP pathways succeed as major clearance route. Three different types of autophagy (literally self-eating) have been described: macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA) (Figure 8). The components degraded by these roots may range from entire organelles, such as damaged mitochondria, to proteins aggregates, soluble peptides or microbes that can be specifically or non-specifically targeted [94].

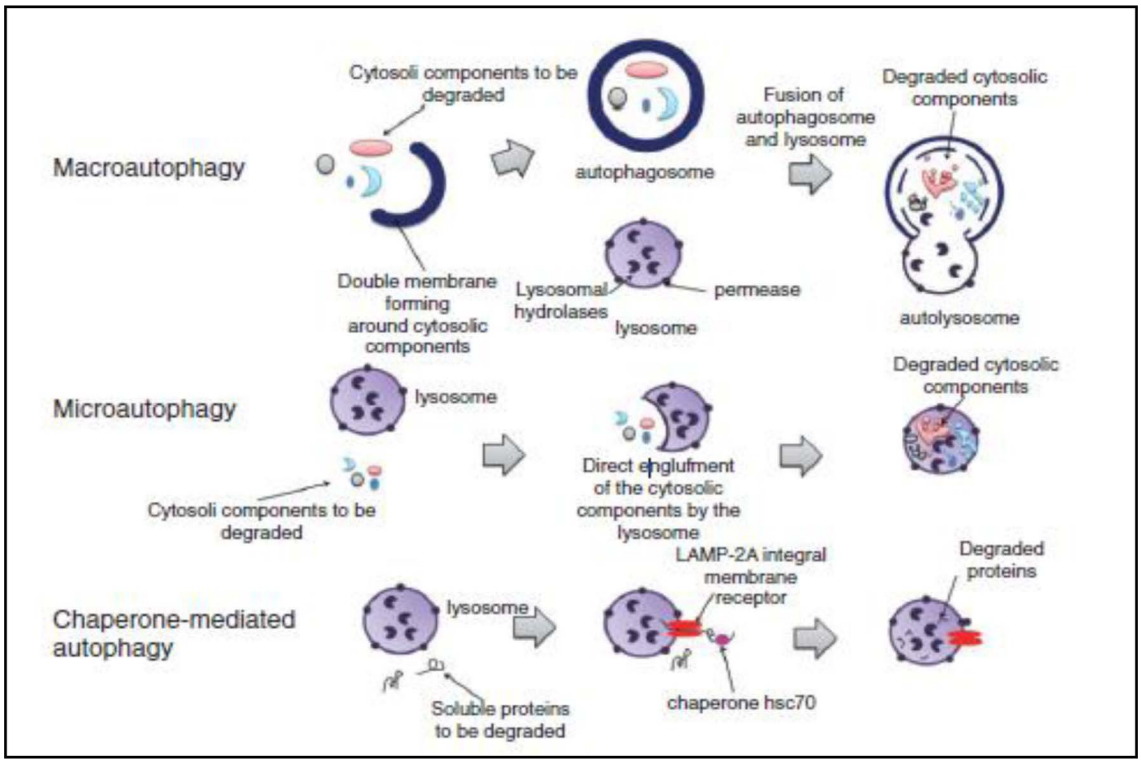

Figure 8: Autophagic pathways: In **macro-autophagy** the cytosolic components to be degraded are included in double layer vacuoles called autophagosome that then fuse with a lysosome, giving rise to an autolysosome, where the intracellular components are degraded by acid hydrolases. **Micro-autophagy** is characterized by the direct engulfment of the intracellular components to be degraded by the lysosome itself. In **CMA** only soluble proteins are degraded by the lysosome, the internalization occurs via the lysosomal-associated membrane protein LAMP-2A receptor and an accessory chaperone, the heat shock cognate protein Hsc70 [95-97].

Each autophagy pathway has individual characteristics, but they all appear to be interconnected and display compensatory up-regulation upon failure of one of

them. Cells respond to a blockage of CMA by activating macro-autophagy in a constitutive manner [98] while CMA is enhanced when macro-autophagy is dysfunctional [99]. The macro-autophagy pathway plays a critical role in the selective elimination of aggregated proteins in mammalian cells, a process known as aggrephagy in which exclusive proteins are required for substrate selection and targeting [88,89].

In summary, a common hallmark in many neurodegenerative diseases is the presence of intra or extra-cellular protein aggregates. All these proteins are degraded by UPS and/or ALS pathways, but persistent high intracellular proteins levels, post-translational modification, mutations or self-aggregation might render them resistant to the UPS-ALS mediated proteolysis, creating an inhibition–accumulation loop that can promote aggregation. Although, the PQC efficiency undergoes an age-dependent reduction and only a small fraction of elderly people develop PD or AD with the presence of intracellular protein aggregates, suggesting that other mechanisms are involved in maintaining a proper protein homeostasis.

2.3. Mitochondrial Dysfunction and Oxidative Stress

Mitochondria are key regulators of cell survival and death [100], play a central role in aging and interact with many specific proteins implicated in genetic forms of neurodegenerative diseases. Mitochondria possess many copies of their own circular DNA (mtDNA) that encode for 13 components of the respiratory chain, 2 rRNAs and 22 tRNAs to support the intra-mitochondrial protein synthesis [101]. Inherited mutations in mtDNA cause a variety of diseases affecting tissues with high energy requirements such as brain and muscles and it is well established that mtDNA accumulates mutations and large-scale deletions with aging that correlate with decline of mitochondrial function [100,102]. Mitochondrial insults, including oxidative damage itself, can cause imbalance between net production and removal of reactive oxygen species (ROS), resulting in ROS accumulation [103]. ROS encompass variety of partially reduced metabolites of oxygen such as superoxide anions, hydrogen peroxide and

hydroxyl radicals which are generated as by-products of cellular metabolism, primarily in the mitochondria. When cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, protein, and DNA may ensue [104]. Several enzymatic and non-enzymatic defence pathways are involved to prevent the accumulation of ROS. These defence mechanisms are not always adequate to counteract the production of ROS, resulting in what is termed a state of oxidative stress [105]. Oxidative stress has been implicated in a wide variety of disease processes including neurodegenerative disorders and is believed to be a major factor in aging [106]. At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest, senescence or cell death [105]. Thus, mitochondrial accumulation of mutations and net production of ROS might contribute to the physiological decline and aging-related neurodegeneration. Many neurodegenerative diseases are characterized by mitochondrial dysfunctions, displaying increased oxidative stress, decreased synaptic activity, reduced energy supply, impaired turnover, defective transport, fragmentation and cell death mediated by activation of the apoptotic cascades upon cytosolic release of cytochrome c and other pro-apoptotic mediators (Figure 9) [107-114].

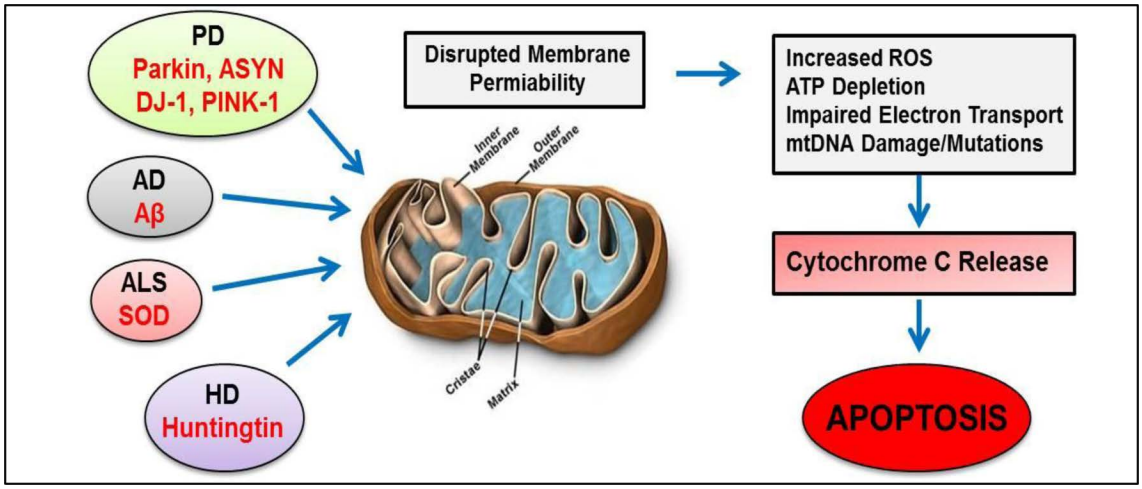

Figure 9: Mitochondrial dysfunctions are associated with several genes related to neurodegenerative disease. Impairment of mitochondrial functions and activity leads to increased production and accumulation of ROS, mtDNA mutations accumulation, cytochrome c release with subsequent apoptosis activation, cell death and neurodegeneration [115].

Therefore, mitochondrial biology holds a central role in the neurodegenerative disorders progression, hence net ROS turnover and energy supply are key events

to maintain a proper neuronal activity and cell survivor is strictly dependent on their integrity.

2.4. Neuroinflammation

Patient brains affected by many different chronic neurodegenerative disorders show increased levels of pro-inflammatory molecules as a consequence of immune response activation [116-122]. The immune response can be divided in innate and adaptive. In the central nervous system (CNS) the innate immune response is mediated by the resident macrophages called microglia. The main function of these cells is to contrast infections caused by bacteria or virus and remove necrotic and apoptotic cells [123]. The effector cells of the adaptive immune response are the T cells. Together with microglia, T cells can help to recover brain damage during neurodegeneration trough a mechanism that still remains unclear [124]. However, impeded microglia activity or persistent microglia activation leading to a systemic inflammation may contribute to neuronal damage (Figure 10).

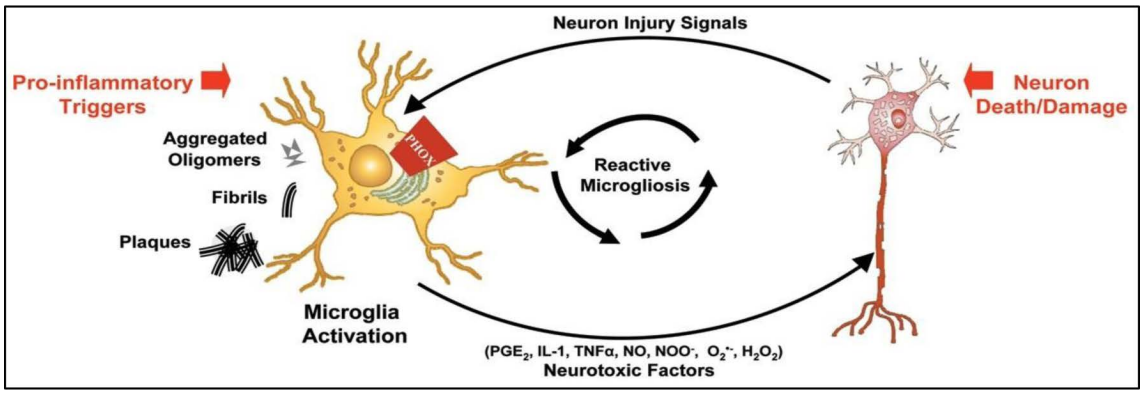

Figure 10: Microglia-mediated neuron damage. In response to disease-specific stimuli, such as protein aggregates, microglia can become deleteriously activated to produce a catalogue of factors, like ROS and cytokines that are toxic to neurons. Neuronal damage/death can also activate microglia to produce these toxic factors. This continual and self-perpetuating cycle of neuronal damage/death followed by microglial activation is commonly called reactive microgliosis and may be an underlying mechanism of the progressive nature of diverse neurodegenerative diseases [125].

In normal conditions T cells activity is repressed by both microglia and neuronal cells, while activated microglia and damaged neurons lose the ability to suppress the inflammation response [126]. The innate immune response cells recruit the adaptive immune cells by secreting various cytokines and by expressing co-

stimulatory molecules. Not only micro-organisms but also endogenous signals coming from damaged or stressed tissue (danger-associated molecular patterns, DAMPs) can activate the immune response within the CNS interacting with the Tool-like receptors (TLRs) expressed by the microglia cells [127]. Some DAMPs, including heat shock proteins, chromatin, ATP and modified or misfolded proteins have adjuvant and pro-inflammatory activity [128]. Notably, both ASYN monomers and tau oligomers are able to induce microglia activation and T cells proliferation [129,130]. Moreover, microglia does not appear to be involved in the removal of protein aggregates or degenerating synapses despite its phagocytic potential [131,132] and preventing the microglia proliferation leads to a delay in the onset behavioural symptoms and prolonged survivor [133]. Animal models of chronic neurodegeneration to deeply study the effects of a persistent inflammation are still missing. However, in the murine prion disease characterized by the accumulation of misfolded protein, microglia is persistently activated as a result of many factors which may include loss of inhibitory contact with neuronal ligands, accumulation of misfolded proteins, presence of neuronal debris and other unidentified routes [134]. It is clear that systemic inflammation is a contributor factor in neurodegeneration [135], thus the use of anti-inflammatory drugs might be a reasonable therapeutic approach to contrast the disease progression.

2.5. Neuronal Loss

Impaired intra–extra cellular processes are the prelude of neuronal cell death in chronic neurodegenerative diseases. Many evidences of activated programmed cell death (PCD) pathways have been collected from post mortem brain analysis of patients affected by neurodegeneration [136-139]. PCD plays an important role in the development of nervous system and three different forms of PCD have been described: apoptotic, autophagic and cytoplasmic [140-143]. These different pathways are activated in response to critical events and neurodegenerative diseases are associated with insults able to activate suicide mechanisms such as accumulation of misfolded proteins, DNA damage, ER

stress, inflammation or mitochondria damage [144]. Apoptotic pathways are largely investigated in neurodegeneration, in particular in diseases characterized by the accumulation of misfolded protein aggregates (Figure 11).

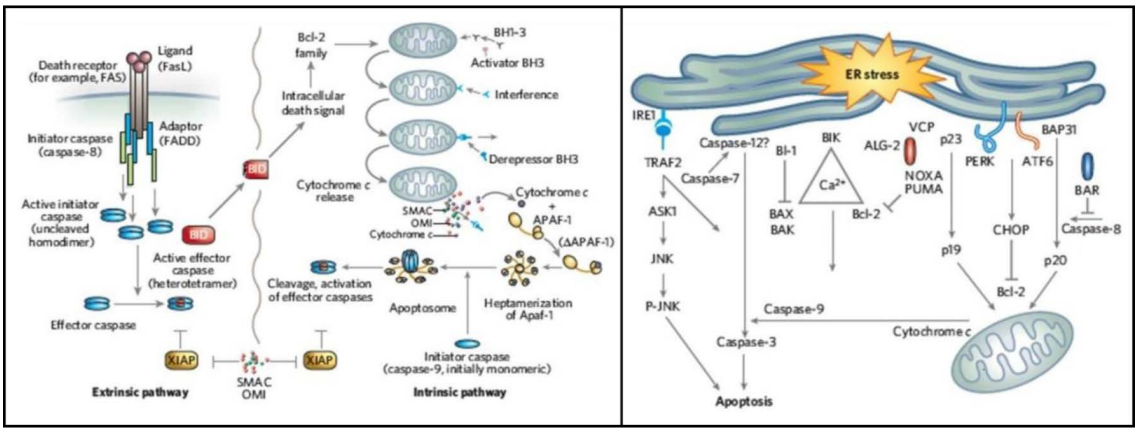

Figure 11: Apoptotic pathways. Three distinct mechanisms are known to trigger apoptotic activation: **(1-extrinsic)** a death receptor–ligand mediated pathway, **(2-intrinsic)** a mitochondrial pathway involving the production of active caspases and **(3-ER mediated)** an endoplasmic reticulum-mediated pathway [144].

The ER is the central organelle for the biogenesis and trafficking of membrane and secretory proteins. It primarily serves as a cellular adaptive mechanism, activating cellular pathways that increase the chaperones mediated protein-folding capacity and reducing the protein influx into the ER by inhibiting gene expression at multiple levels [145]. Proteins that fail to achieve proper conformation are removed from the ER through the ERAD process [146]. Accumulation of misfolded proteins, inhibition of protein degradation or other insults to the ER lead to ER stress, which activates the unfolded protein response (UPR) [147]. Prolonged ER stress or defective UPR mechanisms, result in the activation of apoptotic suicide mechanisms contributing to the progression of neurodegenerative pathologies associated with misfolded proteins [9,91,93,148-150]. Misfolded proteins are also able to aggregate in oligomers and fibrils that interact with many critical cellular targets related to PCD activation. For instance, the disease associated ASYN and Aβ proteins are both able to promote cell death through apoptosis and flogistic processes activation [151-156]. Autophagic PCD represents another important aspect linked to cell death in neurodegenerative disorders. It was initially described as a cell death mechanism but in the latest years many studies revealed how this pathway is essential for

survival when cells are exposed to a wide variety of stress insults including nutrient deprivation, growth factor withdrawal, oxidative stress, infection, and hypoxia [157]. Besides the autophagy pro-survivor function, a persistent activation of this pathways leads to cell death by initiation of a self-digestion process. In progressive neurodegenerative disorders the aggregation and accumulation of misfolded proteins in intra and/or extra cellular aggregates correlates with an abnormal accumulation of intracellular autophagic vacuoles (AVs)—index of impaired autophagy—in degenerating cells. The direct contribution of the disease related Aβ or tau proteins to impaired autophagy is still under investigation, however impaired autophagy initiation in AD brains has been described [158] and in neurodegenerative models autophagy inhibition leads to intracellular Aβ accumulation [159]. Moreover, a defective lysosomal clearance of autophagic substrates due to impaired transport of AVs to lysosomes in association with NFTs deposits has been also established [160,161]. Duplication or triplication of the *SNCA* locus gene is sufficient *per se* to cause PD [64] and ASYN overproduction directly impairs autophagy blocking the formation of omegasomes which are the autophagosome precursors [162]. Specific ASYN post-translational modifications and mutations are also able to inhibit the CMA pathway by blocking the substrate internalization into the lysosome and thus the subsequent degradation [163,164]. Moreover, an indirect autophagy inhibition has also been revealed in the autosomal-recessive form of early-onset PD related to impaired mitochondria turnover [165,166]. Hence, impaired autophagic response might render cells more sensitive to stress conditions inducing cell death [167,168]. In general, complex relationships between autophagy and cell death have been described and in neurodegenerative disorders the specific factors that make autophagy neuroprotective rather than neurotoxic or how impaired autophagy leads to cell death are still enigmatic.

3. The Role of ASYN in Neurodegeneration

3.1. ASYN Structure and Function

The *SNCA* gene is located on chromosome 4q21 and encodes for a 140 amino acid protein called ASYN. ASYN has been defined as "natively unfolded" because it does not have a defined secondary structure in aqueous solution, although it can assume a α-helical secondary structure upon binding to negatively charged lipids such as the phospholipids present on cellular membranes [169]. The ASYN protein can be divided in three distinct regions: **A)** amino terminus region, from 1 to 60 amino acid residues, containing the apolipoprotein lipid-binding motifs, which forms the α-helical structure, **B)** central hydrophobic region, from residues 61 to 95, also called non-Aβ component (NAC), which confers the β-sheet potential and **C)** carboxyl terminus region, negatively charged and unfolded prone (Figure 12) [170].

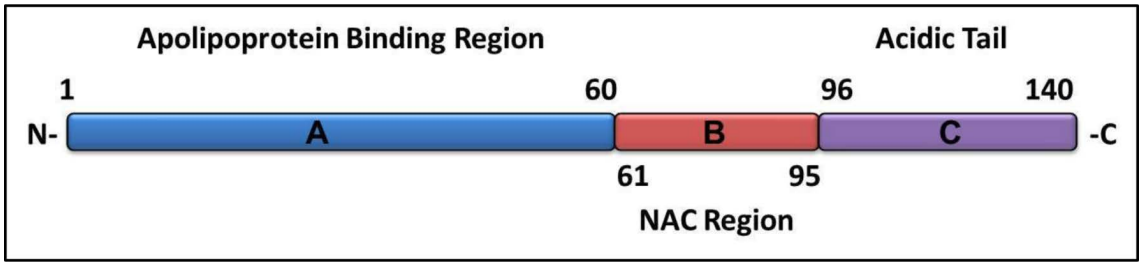

Figure 12: ASYN protein structure and disease-related mutations. The peptide structure can be divided in three main regions, **A)** amino terminus region, **B)** NAC region, necessary for aggregation and **C)** carboxyl terminus region.

Two different ASYN alternative transcript variants exist but their physiological role remains unclear [171-173]. The synuclein family group includes ASYN together with β-synuclein and γ-synuclein and all of them are mainly expressed in the brain, generally localized in the presynaptic terminals. The protein structure is similar for all, except for ASYN which is the only one possessing the NAC region [174]. Some evidence exists about the role of β and γ-synuclein in neurodegeneration [175-177], but a detailed characterization to further assess their potential contribution to neurodegenerative disease progression is still needed. ASYN is highly expressed in the CNS accounting for about 1% of total cytosolic proteins and it is actively transported from the cell body, along axons,

to synaptic termini [178,179]. Small amounts of ASYN protein have been detected also in glial cells [180], while abundant amounts of ASYN protein are detectable in erythrocytes and platelets but the physiological function exerted in these cells remains unknown [181]. During the neuronal cell development ASYN expression is progressively induced depending on the neuronal phenotype, however ASYN localizes to the presynaptic terminals only in a later stages of synaptic development, while it is absent when they first form [182-184]. Moreover, ASYN expression is modulated in critical conditions altering the synaptic plasticity or leading to injury [183,185,186], it is implicated in the size regulation and amount of the presynaptic vesicular pool and might have a chaperone activity to other presynaptic membrane proteins [187-189]. The main function of ASYN appears to be the control of neurotransmitter release by a chaperone-like function, in synergy with CSPα, in the assembly of SNARE complex (Figure 13) [188,190].

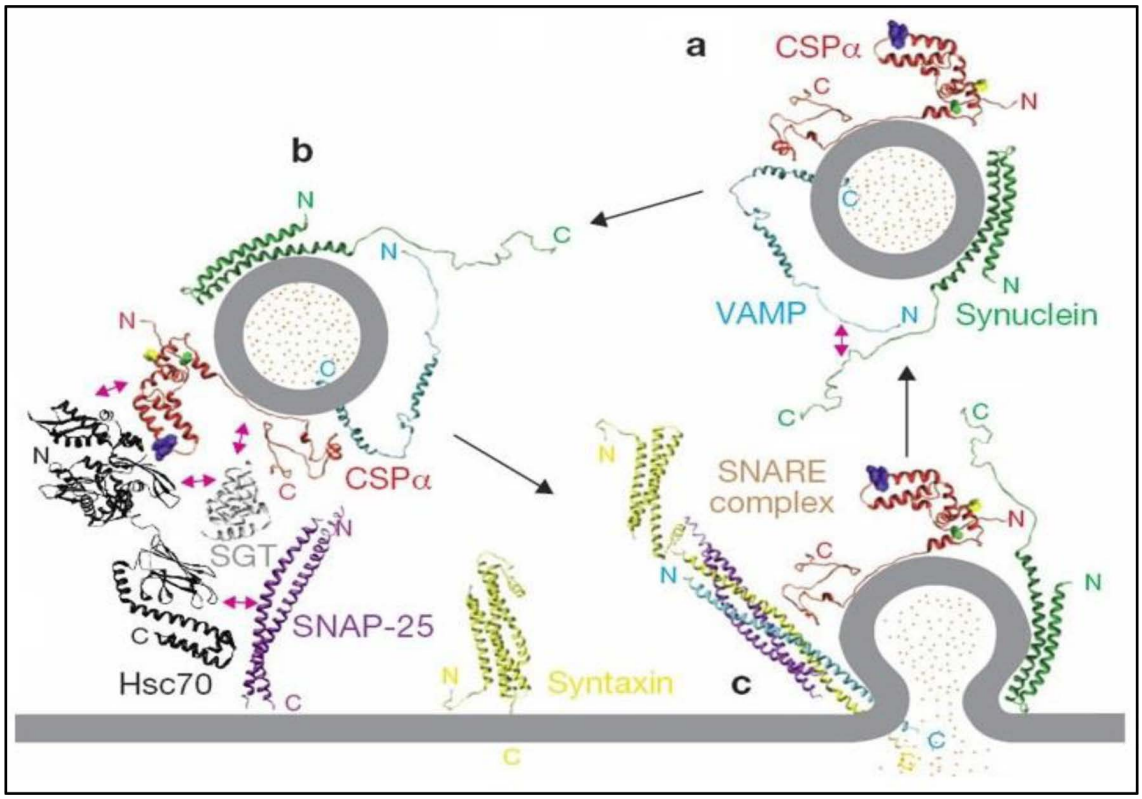

Figure 13: Role of ASYN and CSPα in the synaptic vesicle cycle. a) Binding of the carboxyl terminus of ASYN to the amino terminus of synaptobrevin (VAMP) primes subsequent SNARE complex assembly. **b)** Binding of Hsc70 to SNAP-25 recruits SGT and CSPα to form a chaperone machine that promotes a SNAP-25 conformation compatible for SNARE complex formation. **c)** SNARE complex assembly drives membrane fusion and neurotransmitter release [191].

The SNARE proteins (**S**oluble **N**SF (N-ethylmaleimide-sensitive fusion protein) **A**ttachment protein **RE**ceptor) consist of more than 60 members and are essential for the fusion of synaptic vesicles with the presynaptic membrane [192]. In neuronal cells the formation of the assembled complex is a critical step to mediate the neurotransmitter release through interactions between SNARE proteins localized on vesicles and those on target membranes [193]. Besides the regulation of neurotransmitter release, because of its membrane binding ability and association with synaptic vesicles, it is possible to assume that ASYN might be also involved in endo or exocytosis mechanisms, essential for neuronal function and survival.

3.2. ASYN Implications in Neurodegenerative Diseases

The term synucleinopathies regroups different neurodegenerative disorders characterized by the presence of insoluble fibrillary aggregates of ASYN protein, called LB. The distribution of the pathology at the cellular and regional level is different in each disease. This group of disorders includes PD, many cases of AD (called LB variant of AD), multiple system atrophy (MSA), DLB, neurodegeneration with brain iron accumulation (NBIA) type I, pure autonomic failure (PAF) and a subtype of essential tremor [194,195]. In most cases, synucleinopathies are sporadic diseases caused by multifactorial processes in which genetic, environmental and lifestyle factors culminate in overall risk [196,197]. The first link between PD and genetic defects was proved in 1997 when the mutation G209A in the *SNCA* gene, resulting in an A53T amino acid change in the ASYN protein, was associated with autosomal-dominant familial cases of early-onset PD in Italian and in some Greek kindred [198]. Later, two more mutations in the *SNCA* gene corresponding to A30P and E46K amino acid changes [199,200], together with duplication or triplication of the *SNCA* locus and Rep1 microsatellite expansion, were associated with autosomal-dominant forms of familial PD [64,201-203].

3.2.1. ASYN Expression

The correlation between ASYN protein levels in synucleinopathies pathogenesis arises from the association of *SNCA* gene duplication or triplication and *REP-1* polymorphisms, which leads to increased *SNCA* transcriptional activity, with familial forms of PD. Little is known about the mechanisms involved in *SNCA* transcription regulation. Recently, a signalling pathway involving the ERK/PI3 kinases that mediates the *SNCA* transcriptional activity has been identified [204,205]. Is has also been reported an ASYN-mediated sequestration from the nucleus of the methylation factor Dnmt1 leading to decreased *SNCA* methylation and enhanced transcription creating a feed-forward loop [206]. Epigenetic factors are also involved in *SNCA* transcription regulation and abnormal *SNCA* gene methylation, causing enhanced *SNCA* expression, has been observed in PD brains [207,208]. Familial point mutations in the *SNCA* gene are also involved in *SNCA* expression. It has been observed that the expression of mutant alleles is suppressed over time through mechanisms involving histone methylation and in particular the presence of the A53T mutation enhances the expression of the wild-type allele in a compensatory manner. Nevertheless, the total levels of ASYN mRNA are above the normal controls suggesting, in this case, that enhanced wild-type ASYN expression is responsible for the disease [209-211]. Moreover, in synucleinopathy models, when ASYN is phosphorylated in S129 it preferentially localizes to the nucleus, reducing the histone acetylation affecting the general mechanisms of gene transcription and promoting neurodegeneration [212-214]. There is a general agreement that ASYN causes neurodegeneration through a gain-of-function when it exceeds a certain level. Concordantly, ASYN protein levels are increased in the substantia nigra of aging brains and a significantly increased *SNCA* expression was observed in surviving nigral neurons coming from PD brains [215,216]. However, in synucleinopathies models, neurotoxic effects can occur upon a severe reduction of ASYN protein levels [217,218]. Hence, the loss-of-function as consequence of the endogenous ASYN sequestration by oligomerization and inclusions formation causing neuronal damage and in particular, impaired

synaptic neurotransmitter release, might be involved in synucleinopathies progression as well.

3.2.2. ASYN, Aggregation Mechanisms and Clearance

The presence of LB in synucleinopathies is the final step of ASYN self-aggregation process, which starts with intracellular ASYN accumulation and gives rise to dystrophic neurites, defined as aberrant neuritic sproutings, swollen dendrites, and/or swollen axons [219]. The aggregation process starts with the formation of soluble oligomeric forms of ASYN protein aggregates in β-sheet structure (protofibrils) that gradually become insoluble and evolve into fibrils forming mature LB (Figure 14), although the exact neurotoxic soluble and/or insoluble oligomeric species still remains to be identified. Persistently increased intracellular ASYN protein level can promote the formation of ASYN containing aggregates [220]. ASYN oligomerization is also enhanced by interaction with other proteins, fatty acids and post-translational modifications such as phosphorylation, oxidation, nitrosylation, glycation or glycosylation.

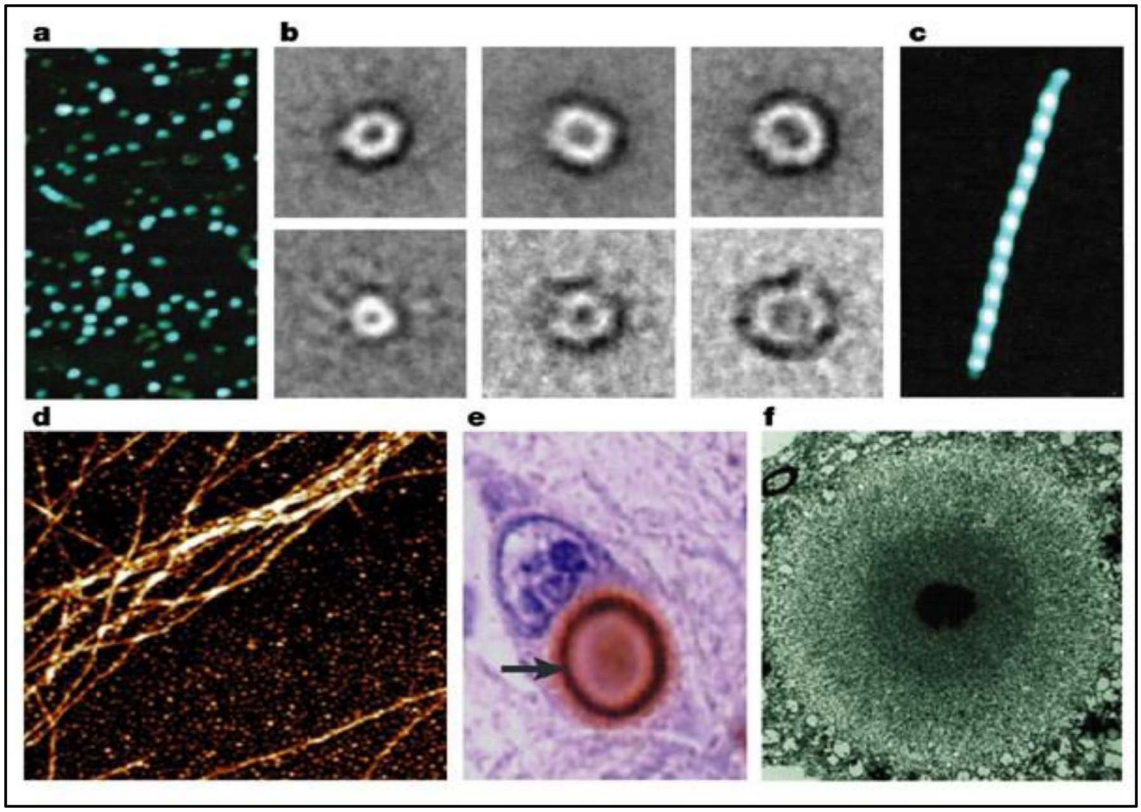

Figure 14: Electron microscope pictures of ASYN aggregates. a-b) Protofibrils round or elliptical shaped. **c-d)** Fibrils in β-sheets structure amyloid like. **e-f)** Round shaped LB inclusions ASYN containing (ASYN immunohistochemistry is indicated by the arrow in e) [221].

Phosphorylation is the most studied modification as in LB there is a conspicuous amount of ASYN hyperphosphorylation at S129 [222]. The effects of S129 hyperphosphorylation on ASYN aggregation and cytotoxicity are still controversial [223-227] but currently it seems that hyperphosphorylation in S129, together with S87, occurs mainly in mature LB [228]. Besides, ASYN phosphorylation in these specific epitopes leads to reduced axonal transport speed, as observed for the A30P and A53T mutants, which is a plausible explanation for the formation of perikaryal and neuritic aggregates [179]. ASYN oxidation is another important modification causing oligomerization. Metal ions, dopamine and mitochondrial dysfunctions leading to increased ROS levels all drive to ASYN oxidation and oligomerization [229-235]. ROS are also associated with the peroxidation of cellular membrane lipids and lipoprotein. One of the most important products of lipid peroxidation implicated in the pathogenesis of many neurodegenerative disorders is the highly reactive aldehyde 4-hydroxy-2-nonenal (HNE) [236-239]. ASYN oxidation mediated by HNE generates toxic stable soluble oligomers and inhibits their conversion into insoluble fibrils supporting the soluble oligomers toxicity theory [240]. ASYN nitration, similarly to oxidation, induces comparable effects promoting fibril formation and within the LB there is an extensive accumulation of nitrosylated ASYN [241,242]. Another significant ASYN modification is the carboxyl terminus truncation that generates aggregation-prone fragments [243,244]. Calpain is one of the most important calcium-dependent enzymes involved in the ASYN cleavage, both proteins localizes at the presynaptic terminals [245,246] and increased intracellular calcium influx mediated by ASYN oligomers could generate a feed-forward loop between calpain-mediated ASYN cleavage and oligomeric species formation [247,248]. ASYN oligomerization can also occur upon interaction with other proteins or fatty acids. The most notable protein-protein interactions are with tau and synphilin-1 that directly promote ASYN aggregation enhancing the formation of insoluble inclusions [249-251]. Polyunsaturated fatty acids interact with ASYN as well, enhancing oligomerization and neurotoxicity [252,253] and sequestration of the arachidonic

fatty acid away from the SNARE complex by ASYN has an inhibitory effect on neuronal transmission [254]. In synucleinopathy models based on ASYN variants lacking the NAC region, essential for aggregation, no toxic effects have been reported [255]. Moreover, protective effects have been observed in synucleinopathy models upon treatment with reagents preventing or neutralizing ASYN oligomers [256,257] and upon overexpression of heat shock chaperones which assist the refolding of aggregation prone proteins [258,259]. Thus, contrasting the formation of cytotoxic ASYN oligomeric species might be a plausible therapeutic approach to treat patients affected by synucleinopathies.

Within the cellular environment the appropriate intracellular protein turnover is maintained by the UPS, CMA and macro-autophagy pathways, all involved in ASYN degradation. Soluble unfolded ASYN monomers are targeted for the UPS system and the CMA, whereas ASYN oligomers and ASYN containing aggresomes are targeted for macro-autophagy and lysosomal degradation [164,260-262]. LB are mainly composed by highly ubiquitinated ASYN protein, but also proteasomal subunits, ubiquitinating and de-ubiquitinating enzymes and proteasome activators are abundantly present in these aggregates, asserting an involvement of the UPS pathway in the clearance of ASYN [263-267]. The first evidence of UPS impairment was observed in the substantia nigra of PD brains where the proteasomal activity was significantly decreased in comparison with age-matched controls [268,269]. This evidence correlates with decreased gene expression of several proteasomal subunits only in the substantia nigra and in the cortex of patients, while in other brain regions the expression was unchanged or even increased [268,270-273]. In synucleinopathy models a direct inhibitory effect of ASYN on proteasome activity has been reported in a wide number of studies. Persistently increased intracellular ASYN protein levels leads to impaired proteasome function creating a vicious circle between UPS impairments and ASYN accumulation [154,274-280]. Besides, induced proteasome inhibition elicits a dose-dependent neurodegeneration with formation of ubiquitin and ASYN positive inclusions [281-283] and acute UPS inhibition was shown to induce compensatory clearance pathways by up-regulating autophagic flux

[284,285]. Moreover, by genome wide association studies (GWAS), mutations in PARK2 (an E3 ligase also involved in the proteasome activity) and PARK5 (an ubiquitin hydrolase) represent a risk factor for monogenic forms of PD [74,286]. However, some patients exhibit a normal or enhanced proteasome activity in the brain regions affected by the presence of LB, suggesting that region-specific perturbation of proteasome function is unlikely to explain the cause of neurodegeneration [287,288].

Together with the UPS system, CMA and macro-autophagy are the two compensatory pathways involved in the ASYN clearance that ends with the degradation of the targeted proteins inside the lysosomes. In CMA the lysosome internalization of targeted proteins occurs via the lysosomal-associated membrane protein LAMP-2A receptor [95-97]. Mutant A53T and A30P ASYN were shown to strongly bind the CMA receptor LAMP-2A but unable to be internalized for degradation, thus acting as CMA inhibitors [163]. A similar inhibitory effect on LAMP-2A occurs upon dopamine-induced conformational changes in wild-type ASYN [164]. However, contrasting results exist in relation to the levels of the CMA receptor LAMP-2A. These were found to be reduced in the substantia nigra and amygdala of PD patients and increased in the temporal cortex of DLB patients [289,290]. Defective CMA was also observed in familial PD patient’s brains carrying mutant LRRK2 protein which interfere with the organization of the CMA translocation complex that could results in ASYN accumulation and other PD related proteins degraded by this pathway [291]. On the other hand, the involvement of macro-autophagy in synucleinopathies was first proved by ultrastructural examination of neurons in the substantia nigra of PD patients revealing accumulation of AVs, which is consistent with either overproduction or impaired vacuoles turnover [292]. This finding was supported by increased autophagosome markers and decreased lysosomal markers in degenerating neurons, validating the presence of abundant and dysfunctional autophagosomes [289,293-297]. Persistent increased intracellular ASYN levels are able to inhibit macro-autophagy in a very early stage, blocking the formation of omegasomes, which are the autophagosome precursors, via Rab1 inhibition

and Atg9 mislocalization [162]. Moreover, lysosomal dysfunctions also affect the secretion of ASYN via exosomes [298], which could have implications for the cell-to-cell spreading and disease progression [299]. Collectively, all these findings show the ability of ASYN to inhibit all the clearance pathways involved in the maintenance of a proper intracellular protein turnover, an impairment that might be crucial in the pathology progression. Thus, restoring the proper PQC system activity could be a plausible approach to arrest or reduce the disease spreading.

3.2.3. ASYN and ER/Oxidative Stress

In synucleinopathy models, ER stress together with Golgi fragmentation leading to cell death is an early event that occurs in concomitance with the appearance of ASYN soluble oligomeric species [300,301], arguing that the primary target of ASYN-mediated neurotoxicity might be the ER-Golgi compartment. The ER-to-Golgi trafficking seems to be impeded through a direct ASYN-mediated mechanism, blocking the vesicle docking and fusion at a pre-Golgi step and inhibiting the assembly of the ER/Golgi SNARE complex [302]. The proteins involved in the ER-to-Golgi vesicular trafficking (RABS) resulted to be the major class of modulators of ASYN toxicity [303]. Hence, in synucleinopathy models overexpression of RABS involved in the synaptic vesicular functions, in particular RAB1, prevent the dopaminergic toxicity to which neurons are very sensitive [304,305].

The degeneration of dopaminergic neurons in the substantia nigra correlates with impaired mitochondrial activity leading to increased oxidative stress and consequent cell death. Accumulation of point mutations and deletions in mtDNA, causing mitochondrial dysfunctions, have been observed in the brain of PD patients [306], while several mtDNA polymorphisms and haplotypes are associated with the risk of PD onset [307]. In synucleinopathy models ASYN directly interacts with the inner mitochondrial membrane causing mitochondrial fragmentation, activity impairment, increased ROS production and cell death due to the activation of the apoptotic cascade mediated by the mitochondria release of cytochrome c [156,308,309]. Moreover, by GWAS studies, mutations in genes

required for the mitochondrial activity causing mitochondrial dysfunctions are associated with familiar forms of PD [310]. In detail, parkin and PINK1 proteins are associated with the mitochondrial outer membrane and prevent cell death by inhibiting cytochrome c release and caspases activation [311,312], mutations or deficiency cause oxidative stress, selective complex I impairment and altered synaptic functions [313]. DJ-1 is mostly localized in the cytosol, but also in the nucleus and associated with mitochondria [314]. Its biological implications are extremely diverse, it can act as a negative apoptosis regulator [315] or a potential ROS scavenger sensor activating neuroprotective pathways [316]. Mutations in DJ-1gene are associated with increased oxidative stress and apoptosis [317]. HTRA2 is a mitochondrial quality control protein and when released in the cytosol acts as a pro-apoptotic factor [8], mutations induce mitochondria swelling and decreased membrane potential [318]. Finally, LRKK2 is a protein largely present in the cytoplasm but also associated with the mitochondrial outer membrane. PD patients carrying the G2019S mutation exhibit reduced mitochondrial membrane potential and total intracellular ATP levels accompanied by increased mitochondrial elongation and interconnectivity [319], suggesting an important role in the regulation of mitochondrial homeostasis. Furthermore, specific inhibitors of the mitochondria complex I transport chain, such as pesticides and MPTP (1-methyl-4-phenyl-1,2,3,6-teteahydropyridine), are also able to induce neurological changes similar to PD, reconfirming the key role of mitochondria activity and oxidative stress in the disease progression [310]. Hence, restoring the normal mitochondrial activity might be helpful to contrast the progressive neuronal loss observed in patient brains.

3.2.4. ASYN, Cytoskeleton and Synaptic Activity

The cytoskeleton is a cellular scaffold composed by a network of microtubules and plays important roles in both intracellular transport, such as the movement of vesicles and organelles, and cell division. ASYN has been shown to bind and modulate actin polymerization, thus indirectly affecting intracellular trafficking [320]. A direct interaction between ASYN and microtubules has been reported in many studies [321] but weather this interaction leads to enhanced or

decreased tubulin polymerization is still unclear as conflicting results have been reported [322,323]. An indirect negative effect on tubulin polymerization can be related to increased ASYN-mediated tau phosphorylation leading to defects on the microtubular network assembly [324,325]. Notably, in the case of tubulin depolymerisation, free tubulin monomers are able to initiate and promote ASYN fibrils formation [326]. In this context, axonal transport defects preceding neurodegeneration can be also related to ASYN-mediated abnormal cytoskeleton assembly as shown in synucleinopathies models [327].

Given the physiological role of ASYN in the regulation of neurotransmitter release, many models have been created to study the synaptic effects of ASYN-induced neurotoxicity. The pathological repercussions reported describe a general impairment in the neurotransmitter release pathway preceding the synaptic and neuritic degeneration. In detail, ASYN overexpression was associated with loss of presynaptic proteins, decreased neurotransmitter release, redistribution of SNARE proteins, swelling of synaptic vesicles and inhibition of vesicle recycling [327-330]. Still, the exact cascade point in which ASYN initiates its neurotoxicity is still unknown. However, since the synaptic neurotransmitter release is a calcium-dependent mechanism, increased intracellular calcium influx mediated by the ASYN oligomers that form pores in the cellular membrane might be the starting point [247,248]. In a similar manner, synaptic vesicles can be damaged by ASYN oligomers causing cytosolic neurotransmitter release that could lead to increased oxidative stress and cell death [331,332]. Here, again, cells undergo a self-feeding cascade between ASYN oligomers, axonal transport defects, calcium perturbation and cytosolic neurotransmitter leakage leading to neurodegeneration. Moreover, as mentioned, the calcium-mediated calpain activation that originates carboxyl terminus truncated aggregation-prone ASYN fragments could enhance this potential vicious circle.

3.2.5. ASYN Cell-to-Cell Propagation

Initially ASYN was thought to be an intracellular protein, but now it is well known that it can be secreted and uptaken by neighbouring cells. The

secretion mechanisms are still unclear but appear to occur via a non-classical secretory pathway through exosomes release upon calcium influx [333-335]. On the other hand, ASYN uptake is dependent on cell type and on ASYN species. Oligomeric ASYN aggregates appear to be easily taken up acting as seeds for the fibrillization of endogenous monomers [247,336-338] supporting the “prion-like propagation” theory. Interestingly, ASYN propagation has been associated with mitochondrial dysfunction as upon intragastric pharmacological inhibition of mitochondria complex I,ASYN gradually spreads from enteric cells to neuronal cells [339], consistent with early enteric ASYN pathology in PD patients and *in vivo* models [340,341]. Importantly, secreted ASYN can induce inflammatory response by glial cells causing cell death and thus enhancing the neurodegeneration [342-344].

3.3. ASYN Physiopathology in Yeast Models

Yeast models are largely used to study ASYN induced cell death. The yeast *S. cerevisiae* does not possess any ASYN ortholog, nevertheless this organism results to be a powerful tool to uncover important aspects for the physiopathology of synucleopathies. The binding of ASYN to the yeast plasma membrane is mediated by the interaction between the detergent-resistant membrane domains, known as lipid rafts, and the N-terminal ASYN region, containing the apolipoprotein lipid-binding motifs. This interplay is fundamental for the subsequent inclusions formation, which occurs between the ASYN central hydrophobic regions (NAC) interaction [347-351]. Hence, mutations that disrupt the N-terminal α-helix formation, such as the ASYN diseased related A30P mutant or other mutations that prevent the N-terminal acetylation, preclude the membrane interaction and the following self-aggregation process [348-350]. When expressed in yeast, the human wild-type ASYN and the mutants A53Tor E46K are delivered to the plasma membrane trough the secretory pathway where they begin to accumulate [345,346]. The aggregation process starts with the appearance of small nuclei that continue to grow over time and then translocate to the cytoplasm forming larger aggregates [345,347]. Because of its decreased

membrane binding ability, the A30P mutant is mainly diffused in the cytoplasm and does not form any inclusions. However, it can be forced to produce intracellular aggregates by increasing the lipid synthesis or providing the necessary seeds by co-expression with the wild-type ASYN [346]. Moreover, some of these larger inclusions are Thioflavin-S positive indicating an aggregation status in β-sheets similar to the LB in PD brains, whereas other inclusions represent ASYN induced aggregates of cytoplasmic vesicles (Figure 15) [305,347,348].

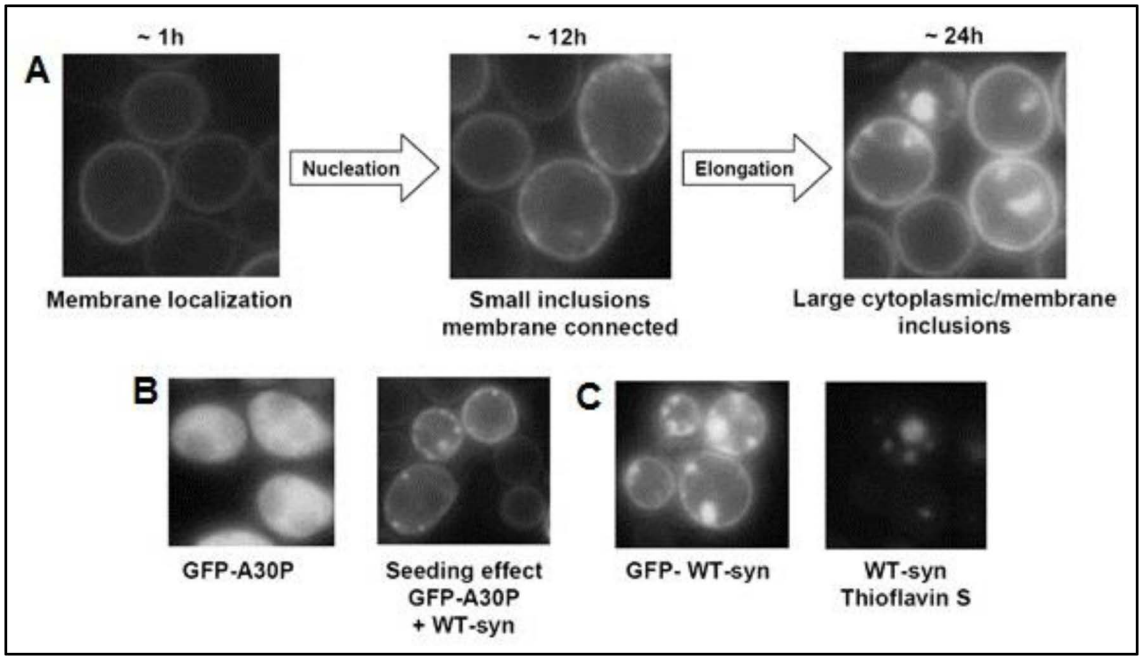

Figure 15: A) Expression of *α*-synuclein in the yeast *S. cerevisiae*. A) At the beginning, wild-type ASYN starts to bind the plasma membrane, small nuclei associated to the plasma membrane start to appear after 12 hours that continue to grow over time forming bigger cytoplasmic inclusions. **B)** A30P mutant is located in the cytoplasm and can form inclusions on the nuclei provided by wild-type ASYN. **C)** Thioflavin-S staining confirms the presence of β-sheeted ASYN aggregates [349].

The exact mechanism that initiates ASYN self-aggregation process is still unclear and besides the membrane binding ability it might involve other post-translational modifications such as phosphorylation, nitration or C-terminal truncation [242,350-352]. In addition, enhanced oxidative stress seems to trigger ASYN oligomerization [353]. In *S. cerevisiae* cytotoxic effects have been observed upon expression of the human wild-type ASYN and the A53T or A30P mutants [354], while cytotoxic effects caused by the expression of the E46K mutant have been reported only in *S. pombe* [346]. In yeast models the ASYN

mediated cytotoxicity seems to be more dependent on the genetic background, the expression levels and the membrane binding capacity rather than on the inclusions formation, as in some strains the presence of intracellular aggregates does not affect the cell growth ability [347,354-356].

As observed in PD patient's brains, the cytotoxic effects mediated by the expression of the human ASYN in yeast models are related to vesicle trafficking, PQC system defects, oxidative stress enhancement and cell death. To reach the plasma membrane from the nucleus through the secretory pathway, the first intracellular yeast compartment encountered by ASYN protein is the ER. The earlier outcome due to ASYN expression is a block in the ER-to-Golgi vesicular trafficking that causes ASYN accumulation within the ER and impaired docking and fusion of ER-derived vesicles to the Golgi membrane [304]. The consequence of this block is the accumulation of ER-derived vesicles near the plasma membrane and defects in the ER retro-translocation of misfolded cytoplasmic proteins for proteasomal degradation through the ERAD process causing ER stress and PQC impairment [304]. Furthermore, additional toxicity is given by hindered endocytosis and secretory pathways because of the obstruction of post-vesicles internalization and vacuoles fusion defects [354,357]. The involvement of PQC molecular chaperones has been observed using yeast strains lacking individual chaperones where the inclusion formation was enhanced when compared with the wild-type strain [357]. Results were further reconfirmed in other studies showing a protective effect against ASYN induced cytotoxicity by chaperones overexpression or pharmacological activation of the heat-shock response [309,345,358], resembling the results obtained in other synucleinopathies models [259,359-361]. Notably, in yeast cells, ASYN expression itself is able to induce the transcription of heat-shock proteins, however this induction is not strong enough to be protective when compared with the response triggered by thermal stress or pharmacological treatments [345]. An involvement of the UPS in yeast models was already evident in the first study of yeast PD model, since many ASYN intracellular inclusions were ubiquitin positive and proteasome impairment was also observed to be related with ASYN

overexpression [354] as the result of an altered proteasome composition in addition to a general decreased protein synthesis [274]. The UPS involvement was then reconfirmed using specific proteasomal inhibitors or proteasomal mutant yeast strains proving that proteasomal inhibition correlates with an enhancement of ASYN accumulation and inclusions formation [345,347,355]. To compensate the UPS failure or insufficient proteasomal activity, misfolded proteins are processed by the autophagic pathway and in yeast, like in mammalian cells, the activation of autophagy by the TOR inhibitor rapamycin leads to a significant reduction of ASYN inclusions [347]. Therefore, contrasting results exist about the role of autophagy in ASYN yeast models as other studies reported an increased ASYN mediated cytotoxicity upon autophagy induction by rapamycin, probably due to increased levels of soluble toxic ASYN conformers [362]. Oxidative stress and cell death are other important aspects of PD physiopathology successfully replicated in yeast models. Metal ions are one of the major risk factors for PD onset because their ability to induce oxidative stress and oligomerization of ASYN [363]. In yeast cells expressing ASYN the treatment with ferrous ions leads to increased cytotoxicity related to increased inclusions formation and oxidative stress markers such as ROS [347,364] together with ASYN-mediated release of typical yeast apoptotic markers such as cytochrome c and phosphatidylserine [309]. Taken together, all these results summarize a self-amplifying killer loop between ER stress, oxidative stress and cell death mediated by ASYN expression that make yeast models a powerful tool to identify compounds and genes for potential therapeutic intervention.

4. The Role of Tau in Neurodegeneration

4.1. Tau Structure and Function

Tau protein, alias Microtubule Associated Protein Tau, is encoded by the *MAPT* gene located on chromosome 17q21 and consisting of 16 exons [365,366]. Tau is a natively unfolded protein especially expressed in neurons where its main function is the stabilization and assembly of microtubules (MT)

through a direct binding with the tubulin protein [367]. In the CNS six tau isoforms exist because of alternative splicing of exons 2, 3 and 10. These isoforms differ by the presence or absence of one or two acidic inserts at the N-terminal domain (exons 2 and 3), and by whether they contain three or four repeats (exon 10) of a conserved tubulin binding motif at the C-terminal region (figure 16) [366,368].

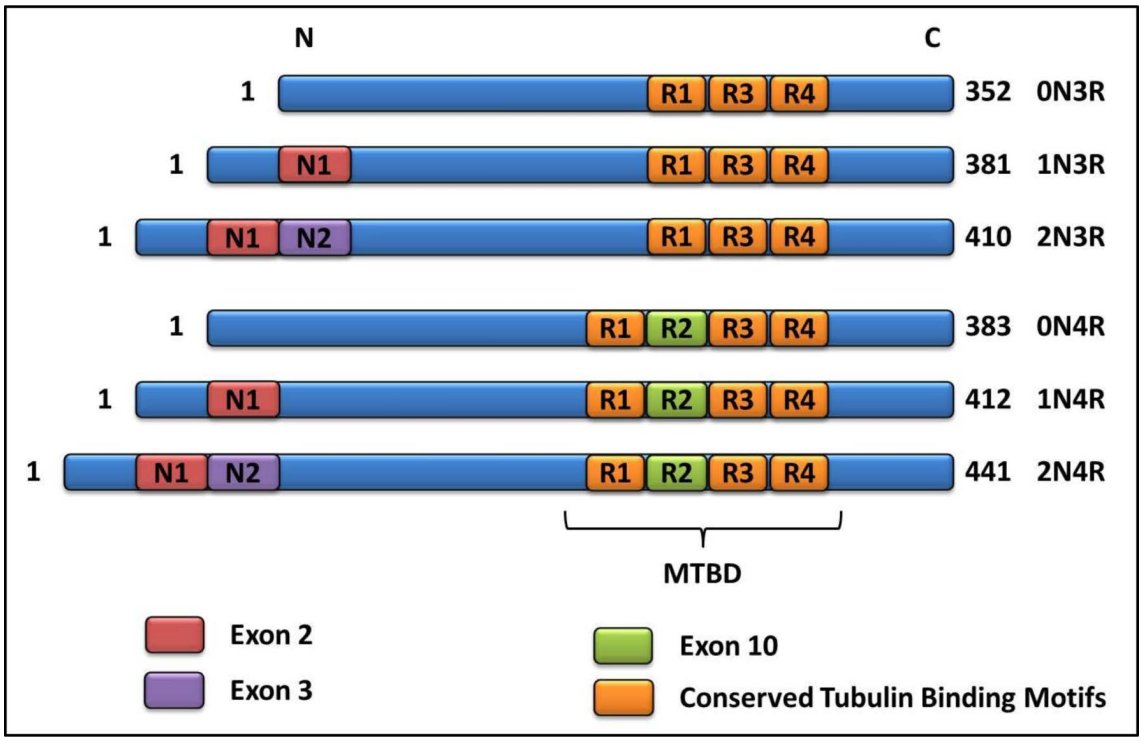

Figure 16: Representation of the six different tau isoforms in the central nervous system and their amino acid lengths.

The microtubule binding domain (MTBD) is the region in charge of binding to MT. The isoforms with four repeats (4R) possess a higher binding affinity when compared to the ones with tree repeats (3R) and in normal human brain the 3R and 4R-tau isoforms are equally expressed [369,370]. The binding affinity of tau for the microtubule (MT) is principally mediated by its serine/threonine phosphorylation status, while hyperphosphorylated tau is unable to interact with the MT and starts to self-aggregate [371,372]. Besides the binding properties to the MT, tau can also interact with actin affecting its polymerization and thus modulating the interaction of actin with MT to achieve the appropriate cytoskeleton assembly [373-375]. Given its physiological function in cytoskeleton polymerization, tau plays an essential role in maintaining the

appropriate cell morphology and, as the MT network is implicated in the axonal transport of molecules and organelles, impaired MT assembly can have important repercussions on the function and viability of neurons [376]. Moreover, tau can interact via its N-terminal region with the plasma membrane and with several proteins involved in the signal transduction, suggesting a dual role of tau both as substrate or activity regulator [377-381]. For instance, tau can be phosphorylated by the Fyn kinase acting as substrate but can also regulate the Fyn activity that is connected with the recruitment of both tau and Aβ within the lipids rafts [382-385]. Because of the large number of tau interactions implicated in many intracellular pathways, ranging from cell morphology to cell signalling, it is still hard to uncover the exact mechanisms that are responsible for tau misfolding, aggregation and cell death.

In the last years several mutations in the *MAPT* gene have been discovered and associated with neurodegeneration. In 1994 the first dominant inherited form of frontotemporal dementia with parkinsonism linked to the chromosome 17q21-22 (FTDP-17) was identified in the region containing the *MAPT* gene, characterized by the presence of tau inclusions in nerve cells and/or glial cells [386]. Later, in 1998, the first mutation in the *MAPT* gene was discovered [387,388] and so far 51 different mutations in the same gene have been identified and associated with FTDP-17 (Figure 17) [62].

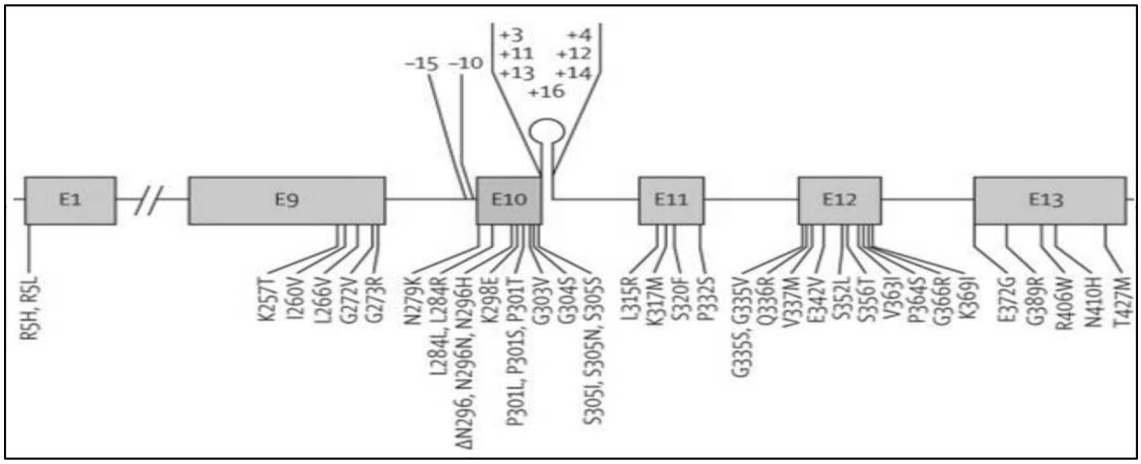

Figure 17: Pattern of identified mutations and respective exons in the *MAPT* gene associated with FTDP-17 [62].

Mutations in the *MAPT* gene account for about 5% of all FTDP-17 cases and can be divided in two main groups. The first group includes the ones affecting the ability of tau to bind the MT promoting tau aggregation, while the second group includes the ones causing alternative mRNA splicing leading to an imbalanced ratio of 3R/4R tau isoforms, resulting in an overproduction of the 4R-tau isoforms [62]. In general, all these mutations seem not to affect the number or the position of tau phosphorylation sites suggesting that hyperphosphorylation is not the main toxic event in FTDP-17. However, some of them might be phosphorylated faster than the normal isoforms [389]. Notably the P301S mutation, that greatly reduces tau ability to promote MT assembly, can cause different syndromes in the same family e. g. frontotemporal dementia in the father and corticobasal degeneration in his son [390]. Furthermore, in the European descendent population the *MAPT* gene possesses two different polymorphisms identified by the inversion (H1) or non-inversion (H2) of ~900 Kb on chromosome 17q21, spanning the entire *MAPT* coding region [391,392]. By GWAS, the haplotype H1 represents a risk factor for a number of neurodegenerative diseases, such as the progressive supranuclear palsy, corticobasal degeneration and idiopathic PD [393-403]. On the other hand, the haplotype H2 is associated with increased expression of exon 3 suggesting a protective effect mediated by the insertion of this exon as the tau isoforms containing exons 2,3 and 10 show a decreased aggregation aptitude if compared with the tau isoforms containing exons 2 and 10 [404,405].

4.2. Tau Implications in Neurodegenerative Diseases

The presence of NFTs mainly composed by hyperphosphorylated tau is a common hallmark in tauopathies, a group of several different neurodegenerative diseases of which AD, ALS and FTDP-17 are the most representative (Figure 18). Tau phosphorylation status plays a central role in the tauopathies etiology which is characterized by abnormally hyperphosphorylated tau that gives rise to NFTs. Tau phosphorylation is regulated by several kinases and phosphatases

which are largely studied as potential targets for therapeutic intervention.

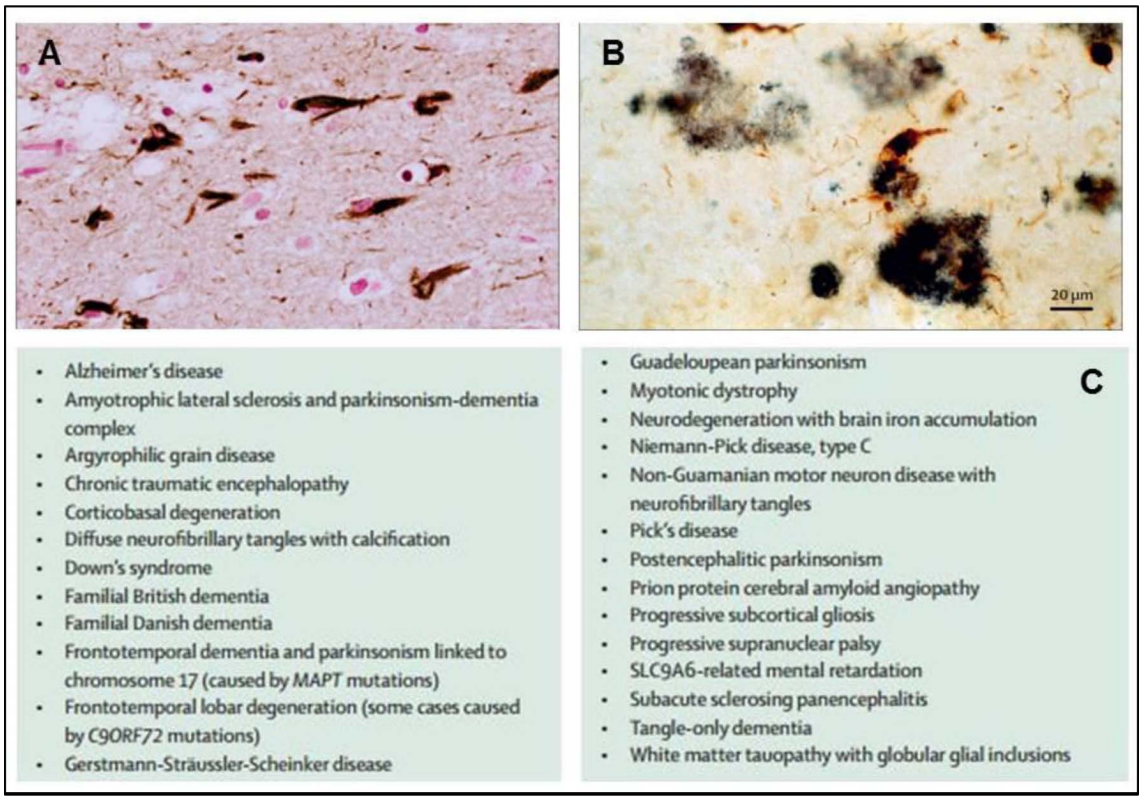

Figure 18: A-B) Neurofibrillary tangles made of tau protein (brown) in chronic traumatic encephalopathy (left) and in AD (right). **C) Neurodegenerative diseases with tau inclusions** [62].

Two major groups of kinases are implicated in tau phosphorylation, the proline-directed protein kinases (PDPKs) and the non-PDPKs. The first group includes glycogen synthase kinase 3 beta (GSK3B), cyclin-dependent kinase 5 (CDK5), MAPKs and stress-activated protein kinases (SAPKs). GSK3B and CDK5 are the most studied ones as both co-localize with MT and directly phosphorylate tau within the intracellular environment [406-411]. Hyperphosphorylation of tau by GSK3B inhibits tau mediated MT assembly and promotes the generation of potential toxic molecules [412-414]. Like GSK3B, hyperphosphorylation of tau by CDK5 promotes dimerization [415] and, interestingly, NFTs have been found in the brain regions containing elevated CDK5 activity [416,417]. Moreover, CDK5 is also able to affects tau phosphorylation status indirectly [418] which occurs, under particular conditions, via a CDK5-mediated inhibition of GSK3B [419]. Among the non-PDPKs group, the microtubule affinity regulating kinase (MARK) and the cyclic AMP-dependent protein kinase (PKA) are the most

important kinases. MARK and PKA phosphorylation motifs are within the tau MTBD, when these motifs are phosphorylated tau is released by the MT and the ability to self-assemble is decreased as well [420,421]. However, the free MT-unbounded phosphorylated tau generated by MARK and/or PKA is the starting point for the formation of hyperphosphorylated tau forms. Thus, the phosphorylation of tau by MARK and/or PKA acts as priming for the subsequent action of CDK5 and GSK3B generating the hyperphosphorylated tau which is the main component of NFTs [422]. At the beginning it was proposed a correlation between neurodegeneration and the presence of NFTs, which were thought to be the primary cytotoxic source [423-425]. However, this theory is still under debate as contrasting results exist about the relation between cell death and NFTs [426], which may even represent a protective mechanism by which neuronal cells try to inactivate other toxic species [71,427].

4.2.1. Tau, Oxidative Stress and Clearance

Besides the presence of intracellular protein aggregates, mitochondrial dysfunctions and oxidative stress are another important aspect of tauopathies. However, the direct repercussion of tau protein on mitochondrial activity is still elusive. What is known about the interaction between tau and mitochondria is that tau redistribution from the axons to the cell body, together with retrograde mitochondria transport and cell death, occurs upon treatment with mitochondrial complex I inhibitor [428]. The retrograde transport is a general process for the elimination of dysfunctional mitochondria by autophagy with subsequent increased ROS levels [429]. In this context it has been proved that increased oxidative stress results in hyperphosphorylated tau species resistant to proteolysis [430], thus the combination of hyperphosphorylation and oxidative stress can be the main event leading to the accumulation of tau aggregates.

NFTs are mainly composed by hyperphosphorylated tau but also mono and poly-ubiquitinated tau forms, ubiquitin and subunits of the E3 ubiquitin ligase complex [59,431-434], suggesting a role of the UPS in tau degradation. The first evidence of an UPS involvement on tau degradation was obtained *in vitro* by showing that tau can be directly processed by the proteasome without

ubiquitination [435]. However, in tauopathies models conflicting results have been produced regarding the UPS role in tau turnover. For instance, in some mammalian cell lines tau was described as a proteasome substrate [412,435-437]. On the contrary, in other cancerous cell lines and in primary neurons where proteasome inhibition correlates with reduced tau protein levels as consequence of constitutive macro-autophagy activation tau is not a substrate [438-442]. Therefore, different forms of tau might follow different pathways in the same cell line, full-length tau is degraded by proteasome while truncated forms are targeted by autophagy [443]. Moreover, hyperphosphorylated and oligomeric forms of tau result in proteasome inhibition [430,444] that could lead to a positive feedback loop which might accelerate the disease progression.

Macro-autophagy and CMA are the other two routes involved in tau clearance that ends with its degradation within the lysosomes. In tauopathy models, a reduction of tau protein levels has been observed upon pharmacological activation of autophagy with rapamycin [442,445,446]. On the other hand, in CMA, tau proteolysis occurs via a selective lysosome internalization through the lysosomal receptor protein LAMP-2A assisted by the molecular chaperone Hsc70 [438]. Some mutant forms of tau seem to be responsible for autophagy impairment, since a dramatic accumulation of autophagosomes and lysosomes arises in tauopathy models expressing the P301L, G272V or R406W mutant tau, creating a self-accumulation loop [447-449].

4.2.2. Tau and Cell-to-Cell Propagation

In human brains of tauopathies, misfolded hyperphosphorylated tau first starts to accumulate in the locus coeruleus and then begins to spread to the entorhinal cortex and other brain regions in an age-associated process with a relatively uniform distribution [450] suggesting a cell-to-cell propagation. This theory was reconfirmed in animal models where human wild-type tau isoforms were injected into mice brains causing the pathology diffusion from the injection site to the neighbouring brain regions without neurodegeneration up to 18 months. In the same animals, upon injection of the P301S mutant tau a massive neurodegeneration was observed after 5 months, suggesting that the toxic tau

species are different from the spreading ones [451,452]. Despite the absence of the secretion signal sequence, monomeric tau has also been detected in the brain interstitial fluid and in the media of cultured neurons causing neurotoxicity by enhancing the intracellular calcium concentration [453-456]. Moreover, in several models, insoluble tau aggregates were taken up via macro-pinocytosis inducing the aggregation of endogenous cytoplasmic tau. Tau aggregates are also directly released into the extracellular environment, however the secretion mechanism is still elusive [457-464].

4.3. Tau Physiopathology in Yeast Models

S. cerevisiae does not possess any tau protein ortholog. Nevertheless, its genome is highly conserved and yeast cells have proven to be a versatile tool to study disease-related molecular processes such as protein misfolding in the field of neurodegenerative disorders [349,465] or to identify novel therapeutic compounds [466]. In yeast models cytotoxic repercussions upon overexpression of the human tau protein have never been reported. However, yeast has been used to study the impact of tau phosphorylation status and oxidative stress concerning the aggregation process. To do this, two particular yeast strains were used to assess the role of Mds1 and Pho85, the orthologous in yeast of mammalian GSK3B and CDK5 respectively, in tau phosphorylation, in combination with several different antibodies against specific phospho-epitopes with pathological significance. Deletion of *MDS1* leads to decreased amount of phospho-tau detected by AD2 and PG5 antibodies. This was an expected observation as the AD2 epitope (S396/404) is a direct target of GSK3B [467]. Since the PG5 epitope (S409) is a direct target of PKA, and not of GSK3B, it has been hypothesized that Mds1 might affect phosphorylation at this site indirectly [468]. On the other hand, deletion of *PHO85* resulted in increased phospho-tau levels recognized by AD2 and PG5 antibodies (Figure 19) in correlation with augmented amount of tau in the sarkosyl insoluble fraction, index of enhanced aggregation rate dependent on tau phosphorylation status [469].

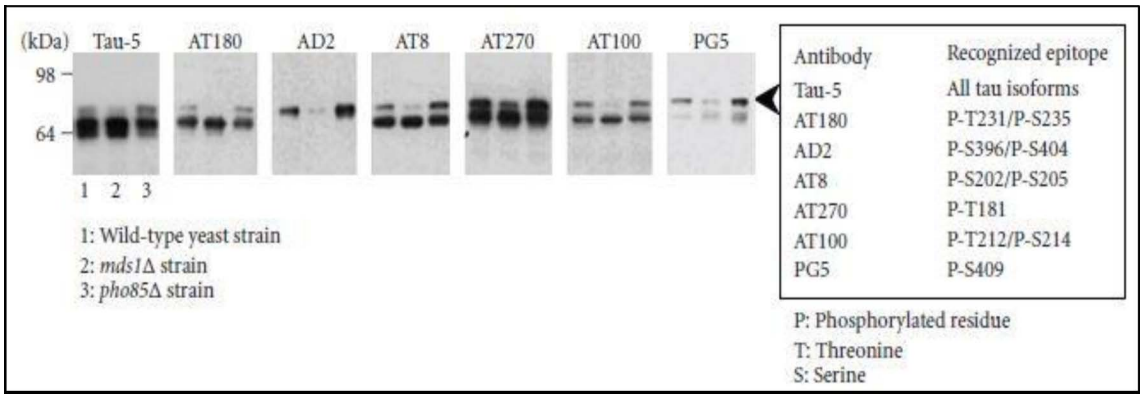

Figure 19: Tau immunoreactivity panel in W303-1A yeast cells. Phospho-tau immunoreactivity detected by AD2 and PG5 antibodies decreased in Δ*MDS1* and increased in Δ*PHO85* yeast strains [465].

In this yeast model, the deletion of *MDS1* and *PHO85* affects tau phosphorylation status in opposite ways as observed *in vivo*, whereas CDK5-mediated inhibition of GSK3B occurs under particular conditions [419]. Furthermore, like proven in tauopathy mammalian models [412-414], an inverse relation between MT binding and tau phosphorylation status was demonstrated, as hyperphosphorylated tau isolated from Δ*PHO85* strain shows a decreased MT binding affinity when compared with the ones isolated from the wild-type and Δ*MDS1* yeast strains [470].

To deeply investigate the relation between tau phosphorylation and its aggregation, several FTDP-17 related tau mutants were expressed in different yeast strains. Notably, the P301L and R406W mutants showed a significant reduction in the phospho-epitope S409 detected by PG5 antibody, in correlation with a decreased aggregation propensity. To further confirm the influence of phosphorylated tau at S409 on its aggregation process the synthetic tau-S409A mutant was then used. This mutant showed a significant reduction in the sarkosyl insoluble fraction together with a decreased AD2 (S396/404) immunoreactivity when compared with the wild-type (2N4R) tau (Figure 20) [471]. Therefore, tau phosphorylation at PG5 and AD2 epitopes seems to be interdependent and phosphorylation at S409 might prime the subsequent phosphorylation at S396/404. The same is observed in AD brains, where phosphorylation at S409 is an early event leading to the formation of NFTs, in which hyperphosphorylation at S396/404 is a pathological feature [472].

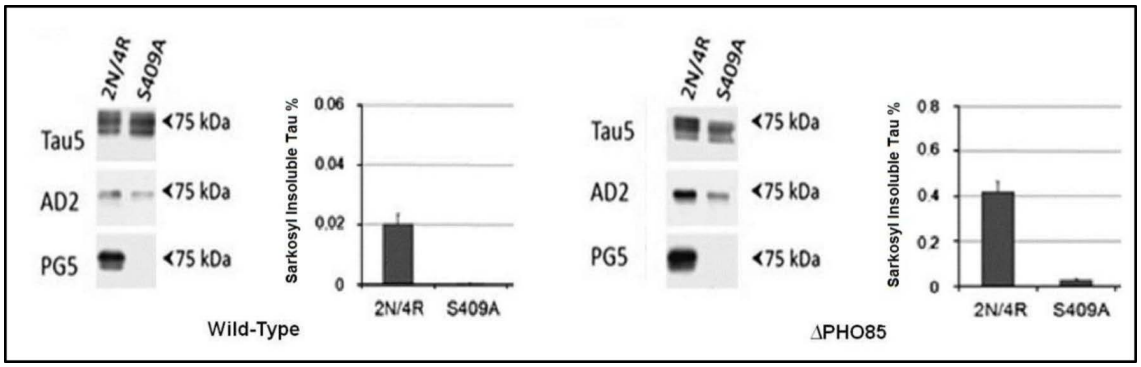

Figure 20: Tau immunoreactivity and aggregation rate. The S409A mutant tau shows decreased AD2 immunoreactivity and decreased aggregation ability by sarkosyl fractionation when compared with the wild-type tau (2N/4R), both in ΔPHO85 and wild-type yeast strains [465].

As previously described, some evidence also exists between oxidative stress, mitochondrial dysfunction and tau pathology. In yeast, the role of oxidative stress on tau aggregation has been investigated in specific mitochondrial deletion mutants or by adding Fe^{2+}, which is a known oxidative stress inducer. In both cases, it a significant increase of tau in the sarkosyl insoluble fraction was observed, through mechanisms that are independent of its phosphorylation status [471]. Taken together, these results recapitulate many important features implicated in tau pathology, including hyperphosphorylation and aggregation. Thus, the yeast model represents a useful tool to further clarify the fundamental cellular processes involved in tau biology and pathology.

5. ASYN and Tau in Neurodegeneration

5.1. ASYN and Tau Interactions

LB and NFTs are hallmarks of synucleinopathies and tauopathies, respectively. However, in patients' brains co-localization of ASYN within NFTs, which are mainly composed of tau protein, and co-localization of tau within LB, which are mainly composed by ASYN protein, was observed, suggesting that a synergistic neurotoxic effect might exist [473-478]. The first evidence of interaction between ASYN and tau was proven in 1999 when a direct binding between the two proteins was revealed [324]. The ASYN binding domain in tau is localized to the MTBD region, while the tau binding site in ASYN resides in

the acidic C-terminal region, in analogy to the tau binding acidic C-terminal region of tubulin (Figure 21). In the same study a competition between ASYN and tubulin for the MTBD in tau was also demonstrated, whereas MT-bound tau was unable to interact with ASYN [324].

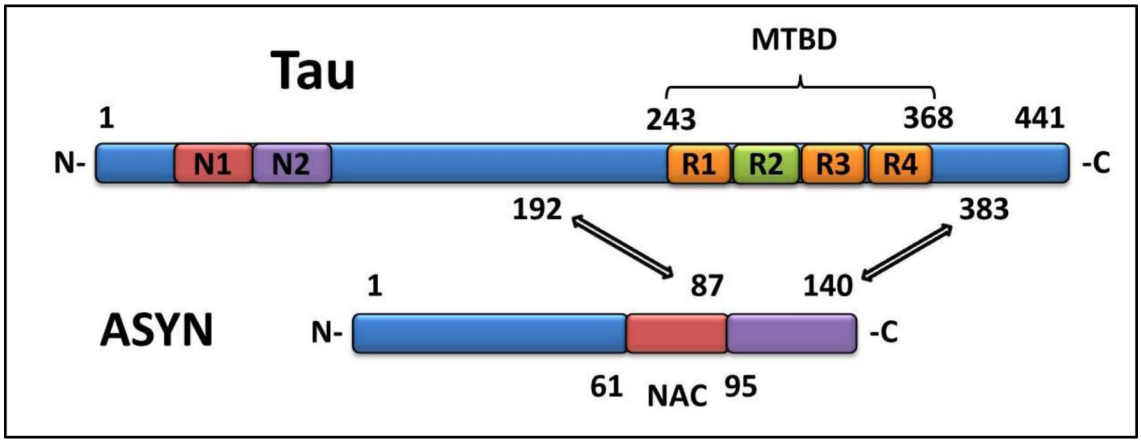

Figure 21: Schematic representation of the ASYN and tau 2N4R binding sites. The tau MTBD containing region and the ASYN C-terminal region are the sites responsible for the direct binding between these two proteins.

The direct binding between these two proteins is abolished by the tau P301L mutation associated with frontotemporal dementia, while the ASYN affinity to tau is decreased by the ASYN A30P disease-related mutation [479,480]. The self oligomerization of ASYN and tau gives rise to the formation of LB and NFTs respectively. *In vitro* assays showed how co-incubation of tau 2N4R and wild-type ASYN promotes the polymerization of both proteins in a time-dependent manner [250]. Similar results have also been reported in mammalian and animal models. In cells overexpressing the human tau 2N4R, recombinant prefibrillized ASYN seeds promote the formation of large round shaped intracellular aggregates, with LB-type morphology, composed of both ASYN and tau proteins [481]. Additionally, double transgenic mice co-expressing wild-type ASYN and P301L mutant tau show ASYN positive and tau positive inclusions in oligodendrocytes that are not observed when wild-type ASYN or tau P301L are solely expressed [250]. Since oligodendrocytes express very low levels of endogenous ASYN and tau proteins, the formation of amyloidogenic Thioflavin-S positive pathological inclusions is due to the interaction of transgenic ASYN and tau proteins that facilitate and promote each other's fibrillization [250,481].

Besides the ability of these two proteins to promote the fibrillization of each other, a synergistic cytotoxic effect upon co-expression of ASYN and tau has also been reported. In mammalian models of synucleinopathy, co-expression of tau enhances ASYN aggregation and toxicity increasing the number of ASYN inclusions per cell and reducing their size [482]. Likewise, in yeast, a synergistic cytotoxicity has been observed in cells that combine the expression of wild-type tau and wild-type ASYN or P301L mutant tau either with wild-type or A53T mutant ASYN [347].

Tau phosphorylation status plays an important role in the etiology of all tauopathies. NFTs are characterized by the presence of hyperphosphorylated tau species and in many studies an ASYN-mediated effect on tau phosphorylation has been reported. ASYN is able to induce tau phosphorylation through indirect mechanisms increasing the activity of several kinases which are directly involved in the tau phosphorylation cascade. In detail, ASYN protein is able to enhance the activity of GSK3B, PKA and c-jun kinases (JNK) increasing the tau phosphorylation status and thus reducing its binding affinity for the MT, facilitating the formation of potential cytotoxic oligomeric species [324,481,483-485]. Moreover, there is an increasing evidence that genetic susceptibility contributes to the etiology of PD. Recently, GWAS confirmed that *SNCA* and *MAPT* (H1 haplotype) genes are two of the main common contributors to idiopathic PD [486-488]. Taken together, all these results reveal that the interaction between ASYN and tau (Figure 22) may be a relevant disease component that enhances the pathological cascade and spreads the neuronal damage in synucleinopathies and tauopathies. However, the molecular pathways by which these synergistic effect leads to neurodegeneration are still unclear. The development of new experimental models to shed light into the mechanisms involved in this synergistic toxicity mediated by ASYN and tau proteins will be useful to further understand the physiopathology of these devastating illnesses.

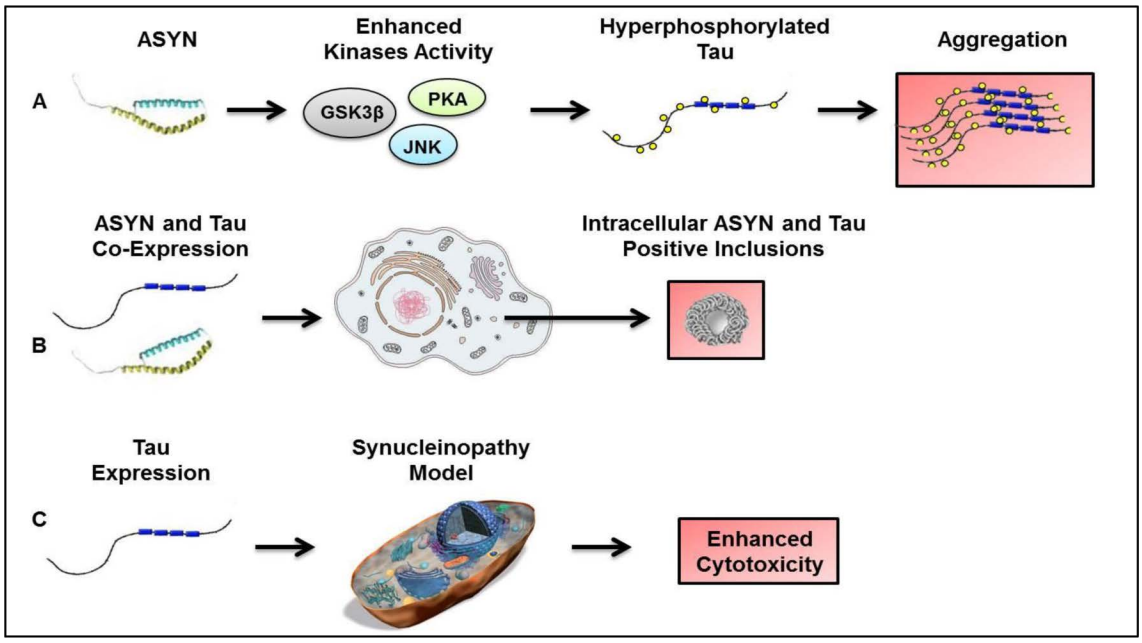

Figure 22: Schematic representation of ASYN and tau interactions. A) ASYN enhances the activity of different kinases involved in tau phosphorylation [324,481,483-485]. **B)** Co-expression of ASYN and tau leads to the formation of intracellular inclusions immunoreactive for both proteins [250,481]. **C)** In synucleinopathy models tau enhances ASYN-mediated cytotoxicity [347,482].

5.2. A Dual Target for Therapeutic Intervention

Synucleinopathies and tauopathies regroup a wide number of neurodegenerative diseases that share common molecular mechanisms. In these disorders the presence of intracellular protein aggregates is a dominant feature and, in some cases, both ASYN and tau co-localize in the same inclusion. During the last century the role of ASYN and tau in the pathology progression has been largely investigated and many molecular pathways leading to neurodegeneration have been identified. The common mechanisms by which ASYN and tau induce neurodegeneration and cell death are related to self oligomerization leading to the formation of potential cytotoxic oligomeric species, calcium dependent cell signalling, mitochondrial activity, cytoskeleton assembly, protein quality control system, cell-to-cell propagation and immune response activation. More recently, many direct and indirect interactions between ASYN and tau and a synergistic cytotoxic effect have also been revealed. Given all these analogies in the physiopathology of synucleinopathies and tauopathies and the cytotoxic synergy of ASYN and tau, these two proteins might represent a dual target to develop

novel therapeutic approaches to treat a wide number of neurodegenerative disorders.

6. Goal of the Project: Identification of Modulators of ASYN and Tau Interaction

Starting around 1960, *S. cerevisiae* was introduced as an experimental system for molecular biology [489] and in 1996 yeast was the first eukaryotic organism for which the complete genomic sequence could be established [490,491]. Most importantly, about 30% of human genes involved in the development of many human diseases have functional orthologous in yeast [492]. Yeast are used for the high-throughput screening (HTS) of libraries due to advantages such as evaluation of compounds in a physiologically relevant environment and immediate negative selection of toxic compounds or those with poor membrane permeability [493]. Moreover, collections of deletion mutant strains are available together with overexpression collections [494-497], providing powerful tools for genome-wide screening (GWS) assays. Despite the absence of a nervous system, yeast cells have been largely used to get insight into neurodegenerative disorders [349,465,498,499], as many of the pathways involved in pathology progression, such as mitochondrial activity, transcriptional regulation, intracellular trafficking and protein quality control, are well conserved among yeast and human [500]. In yeast models, ASYN expression has been widely described as causing toxicity and growth delay, whereas expression of tau has no apparent consequence on cell growth [354,364,470,471]. Co-expression of ASYN and tau was previously promoted in yeast and was reported to be synergistically toxic, although ASYN expression alone resulted in no phenotype [347]. However, a detailed characterization to shed light into the molecular pathways involved in this synergistic cytotoxicity is still missing. Our goal is to create a new yeast model to investigate the synergistic cytotoxic effect mediated by the co-expression and interaction of ASYN and tau proteins. The model will be characterized to identify the molecular mechanism by which the interaction between ASYN and tau is responsible for enhanced cytotoxicity.

After having a powerful model, representative of the toxic synergistic effect between ASYN and tau, we will perform GWS and HTS assays to identify genes and compounds able to modulate the synergistic toxic phenotype caused by the concomitant expression of ASYN and tau. The identification of the target genes and compounds in yeast, represent a valid preliminary screening to shed light in the molecular pathways involved in neurodegeneration leading to cell death. Thus, the identified modulators might be a plausible starting point for further investigations in mammalian models with increased physiological relevance.

Chapter II. Results

1. A Powerful Yeast Model to Investigate the Synergistic Interaction of ASYN and Tau in Neurodegeneration

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A Powerful Yeast Model to Investigate the Synergistic Interaction of α-Synuclein and Tau in Neurodegeneration

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Abstract

Several studies revealed consistent overlap between synucleinopathies and tauopathies, demonstrating that α-synuclein (ASYN) and tau co-localize in neurofibrillary tangles and in Lewy bodies from Alzheimer's and Parkinson's disease patients and corresponding animal models. Additionally, it has been shown that ASYN can act as an initiator of tau aggregation and phosphorylation and that these two proteins directly interact. Despite these evidences, the cellular pathway implicated in this synergistic interaction remains to be clarified. The aim of this study was to create a yeast model where the concomitant expression of ASYN and tau can be used to perform genome wide screenings for the identification of genes that modulate this interaction, in order to shed light into the pathological mechanism of cell dysfunction and to provide new targets for future therapeutic intervention. We started by validating the synergistic toxicity of tau and ASYN co-expression in yeast, by developing episomal and integrative strains expressing WT and mutant forms of both proteins, alone or in combination. The episomal strains showed no differences in growth delay upon expression of ASYN isoforms (WT or A53T) alone or in combination with tau 2N/4R isoforms (WT or P301L). However, in these strains, the presence of ASYN led to increased tau insolubility and correlated with increased tau phosphorylation in S396/404, which is mainly mediated by *RIM11*, the human homolog of *GSK3β* in yeast. On the other hand, the integrative strains showed a strong synergistic toxic effect upon co-expression of ASYN WT and tau WT, which was related to high levels of intracellular ASYN inclusions and increased tau phosphorylation and aggregation. Taken together, the strains described in the present study are able to mimic relevant pathogenic features involved in neurodegeneration and are powerful tools to identify potential target genes able to modulate the synergistic pathway driven by ASYN and tau interaction.

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Introduction

In the last decade, several studies, from genetic features to direct interaction, have revealed a consistent overlapping between synucleinopathies and tauopathies [1–3]. Synucleinopathies consists of a group of disorders in which the pathological hallmark is the presence of insoluble fibrillary aggregates of alpha-synuclein (ASYN) protein, designated by Lewy bodies, in specific brain cells populations. The disorders included in this group are Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [4,5]. The first two mentioned diseases are characterized by the presence of Lewy bodies in the dopaminergic neurons in the substantia nigra of the mid brain region and in the cholinergic neurons in the brainstem, limbic and cortical region, whereas in MSA glial cells are affected showing a high density glial cytoplasmatic inclusions (GCIs) ASYN-positives [4,5]. In most cases PD is a idiopathic disease, involving polygenic mutations, gene interactions and lifestyle, whereas monogenic forms are very rare with only around 20% of cases reporting hereditary features [6–9]. ASYN gene (SNCA) was the first gene discovered to be associated with PD. It was observed that duplication or triplication [10,11] of the SNCA gene, along with three missense mutations (A30P, E46K and A53T), are all related to familial PD [12–14].

Tauopathies represent another group of neurodegenerative disorders also characterized by the aberrant aggregation of specific proteins. The pathological hallmark of tauopathies consists in the presence of neurofibrillary tangles (NFT) labelling positive for tau protein [15,16]. The accumulation of these tangles leads to progressive brain atrophy due to frontotemporal or striatonigral degeneration. Pathologies such as Alzheimer's disease (AD), frontotemporal lobar degeneration (FLD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) are all examples of tauopathies [17]. How NFTs are formed in these diseases is still under investigation. It is known that the paired helical filaments (PHF) of NFT are constituted by hyperphosphorylated tau, mainly promoted by the kinase *GSK3β* which was shown to phosphorylate tau in up to 30 distinct sites [18–20]. In addition to hyperphosphorylation, there are also some tauopathies such as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) in which tau's missense mutation P301L is known as an "aggregation-prone mutation" [21,22].

The overlapping between synucleinopathies and tauopathies involves many aspects. Genome-wide association studies have shown that the microtubule-associated protein tau (MAPT) haplotype H1 displays a significant association with PD [3,23]. Other studies showed ASYN being localized in NFTs, which are mainly composed by tau, and tau localized in Lewy bodies, mainly composed by ASYN [2,24–26]. In fact, in a double transgenic mice expressing human ASYN WT in combination with human P301L mutant tau, these two proteins have been shown to co-localize in the same intracellular inclusions [27]. A direct binding between these two proteins has also been described with disease-related mutations being shown to interfere with this interaction [1,28]. Moreover, it was also described that ASYN can act as a pathological initiator of tau phosphorylation and aggregation, both *in vitro* and *in vivo* [29–32]. Whereas reduction of endogenous tau levels in murine models of Alzheimer's disease has been shown to improve cognitive performance [33–35], tau reduction did not prevent motor deficits in mouse models of PD [36]. These observations rely on the use of two specific animal models and cannot be extrapolated for other models or to the human PD condition without further validation. In addition, implication of tau levels in cognitive function cannot be ruled out. Interestingly, neurite degeneration has been directly correlated with tau protein levels and phosphorylation state in a Drosophila model of PD [37]. Therefore, the mechanism by which ASYN and tau synergistically interact and its role in pathology deserves further investigation.

Yeast models are validated tools for the study of neurodegenerative diseases [38,39]. In fact, both cellular mechanism of action and phenotypical repercussions derived from ASYN expression have been largely studied in yeast [40,41], a non-expensive, easy to handle and with a short replication time model. Whereas ASYN expression in yeast has been widely described as causing toxicity and growth delay, expression of tau in yeast has no apparent consequence on cell growth [42,43]. Neither ASYN nor tau have orthologues in yeast, thus allowing the development of unbiased models of human transgene expression, with no interference or competition from endogenous yeast proteins. Co-expression of ASYN and tau was previously promoted in yeast and was reported to be synergistically toxic, although ASYN expression alone resulted in no phenotype [44]. We wanted to further explore this synergistic phenotype, by making use of distinct expression systems, where protein levels and transgene copy numbers can be more stably maintained. Our goal was to develop a yeast model of ASYN and tau co-expression that will be used for the identification of genes able to modulate this synergistic interaction and hence relevant as future therapeutic targets for human neurodegenerative diseases. Therefore, in the current work, we report the development of different wild type (WT) yeast strains, BY4741 and W303-1A, co-expressing ASYN and tau from episomal or integrative vectors, with the aim of identifying a toxic phenotype caused by the synergistic interaction between the two proteins. Both episomal and integrative strains were further characterized by analyzing the formation of ASYN intracellular inclusions and tau aggregation and phosphorylation states. This detailed characterization allowed the identification of a synergistic toxic effect probably triggered by the presence of ASYN WT cytoplasmatic inclusions and insoluble phosphorylated tau WT. The yeast strains developed in this work are being used as tools for the identification of potential target genes able to modulate the synergistic interaction observed between ASYN and tau in neurodegenerative diseases.

Materials and Methods

Yeast Strains and Media

The following *Saccharomyces cerevisiae* strains were used in this study: BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), W303-1A (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) (T. Outeiro IMM) and the single deletion mutant ΔRIM11 was obtained from the genome-wide yeast deletion collection YSC1053 (Open Biosystems) [45]. All strains were grown in synthetic complete media (SC) which consists of 0.67% yeast nitrogen base (Sigma-Aldrich), 0.067% yeast drop-out mix (MP Bio) and 2% (w/v) carbon source glucose, galactose or raffinose, (Sigma-Aldrich) depending on the experiments, solid media plates also contain 2% agar (BD).

Plasmids

Tau (2N/4R) WT and P301L variants were subcloned from YIpLac128 Gal1 (BIOALVO) into pESC-LEU, a bidirectional high-copy (2μ) episomal vector (Stratagene), under Gal1 promoter using the restriction sites ApaI N-terminal and XhoI C-terminal. ASYN WT and A53T variants were subcloned from pRS426 GPD (T. Outeiro IMM) into the same pESC-LEU vector but under Gal10 promoter using the restriction sites SpeI N-terminal and HindIII/SacI blunt end C-terminal and into pRS306 Gal1 (BIOALVO) using the restriction sites SpeI N-terminal and HindIII C-terminal. BY4741 WT and ΔRIM11 yeast strains were transformed with the episomal vector pESC-LEU harbouring ASYN (WT or A53T) and tau (WT or P301L) alone or in combination. W303-1A yeast strain was transformed with the integrative vectors pRS306 Gal1 harbouring ASYN (WT or A53T) and YIpLac128 Gal1 harbouring tau (WT or P301L) alone or in combination. Both transformation of yeast strains using the lithium acetate method and transformants colony selection were performed as described in [46]. Confirmation of integration was performed by PCR using the following primers: fw_aggagcacagacttagattg and rw_ttggagcctacatagagaac for the pRS306 Gal1 harbouring ASYN isoforms and fw_gcaatcgtcttactttctaa and rw_gcaaatagtctacaaaccag for the YIpLac128 Gal1 harbouring tau isoforms.

Spotting Assays and Growth Curves

Cell growth was analysed on solid selective SC medium to maintain selection for plasmids by spot assay by performing 5x-fold serial dilutions of exponential growing cultures, starting at OD_{600} = 1,0. For this, cells were pre-grown overnight at 30°C with agitation (200 rpm) in selective SC medium, containing raffinose. After this pre-incubation, cells were re-inoculated in the same selective SC medium at OD_{600} of 0,2 and left to grow at 30°C with agitation (200 rpm) until an OD_{600} of 1.0 was reached. Spot plates containing glucose (non-inducing medium) or galactose (inducing medium) were incubated at 30°C or 37°C with images being acquired after 72 h of incubation. Quantification analysis was performed using ImageJ [47] acquiring the intensity of all the spots in each lane from the plates containing galactose and then normalized versus the respective values obtained from the control plate containing glucose. Statistical significance was determined by performing one-way ANOVA with post-hoc Dunnett's test using GraphPad Prism. Growth was also analysed in liquid medium. For this, cells were pre-grown overnight at 30°C with agitation (200 rpm) in selective SC medium containing raffinose. After this pre-incubation, cells were re-inoculated in selective SC medium containing glucose (non-inducing medium) or galactose (inducing medium) and incubated at 30°C or 37°C in 96 well plates. Growth

was automatically monitorized by measuring OD_{600} using a PerkinElmer Victor 3V spectrophotometer.

Immunoblotting

Immunoblotting was conducted following standard procedures [48] with some minor modifications. Yeast cells were pre-grown at 30°C with agitation (200 rpm) in selective SC medium containing raffinose. After this pre-incubation, cells were inoculated at OD_{600} of 0,2 in selective SC medium containing galactose and incubated overnight at 30°C (W303-1A) or 37°C (BY4741 WT and ΔRIM11) with agitation (200 rpm). Cells were harvested by centrifugation, washed in sterile water and pellets were resuspended in 100 µl of 1X SDS sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 70 mM βME, 1% bromophenol blue supplemented with 1x protease inhibitor cocktail (Sigma-Aldrich) and 1x PhosSTOP (Roche) phosphatase inhibitor cocktail). After resuspension, pellets were lyzed by boiling for 5 minutes. 15 µl of each sample was run in a 12% SDS-PAGE gel and blotted onto PVDF membrane. Immunodetection was carried out using the following primary antibodies: Total tau (Polyclonal Rabbit anti-Human Tau, Dako) diluted 1:10000, phospho tau in S396/404 (Mouse AD2 Anti-Tau Protein mAb, BioRad) diluted 1:5000, ASYN (Purified Mouse Anti-α-Synuclein, BD) diluted 1:1000 and GAPDH (Mouse Monoclonal Anti-GAPDH, Ambion) diluted 1:3000, all in in TBST containing 1% milk. The following secondary antibodies were used: Goat Anti-Mouse IgG (H+L)-HRP Conjugate (BioRad) diluted 1:10000 and oat Anti-Rabbit IgG (H+L), Horseradish Peroxidase conjugate (Invitrogen) diluted 1:10000, all in TBST containing 1% milk. Membranes were revealed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and digital images acquired with Alliance 4.7 (UVITECH Cambridge). Quantification analysis was performed using ImageJ [47] and statistical significance was determined by performing one-way ANOVA with post-hoc Dunnett's test using GraphPad Prism.

Sarkosyl Fractionation

Sarkosyl fractionation method was performed as described in [49] with some minor modifications. Yeast cells were pre-grown overnight at 30°C with agitation (200 rpm) in selective SC medium containing raffinose. After this pre-incubation, cells were re-inoculated at $OD_{600} = 0,2$ in 50 ml of selective SC medium containing galactose and incubated for 24 hours at 30°C (for W303-1A) or 37°C (for BY4741 WT) with agitation (200 rpm). Cells were then harvested by centrifugation, washed in sterile water and pellets were resuspended in 500 µl of Extraction Buffer (100 mM Tris-HCl pH 7,9, 250 mM ammonium sulfate, 1 mM EDTA, 10% glycerol, 0,5 mM DTT supplemented with 1x protease inhibitor cocktail (Sigma-Aldrich)). Resuspended cells were homogenized with glass beads for 10 minutes at 4°C, total protein extract concentration was measured by Bradford protein measurement method assay [50] and adjusted to 1 mg/ml (Input). Sarkosyl (Sigma-Aldrich) was added to the lysates to a final concentration of 1% and samples were incubated at room temperature for 5 minutes. Sarkosyl-soluble and insoluble fractions were separated by centrifugation at 35,000 g for 1 h at 4°C. Pellets were washed once with extraction buffer and centrifuged at 35,000 g for 30 min to eliminate residual protein from soluble fractions. After centrifugation, pellets were resuspended in 15 µl of 1X SDS sample buffer and boiled for 5 min, before loading onto a 12% SDS-PAGE gel. Equal volumes of input and sarkosyl soluble fraction were diluted with 2X SDS sample buffer and boiled for 5 min, before loading onto 12% SDS-PAGE gel.

Immunofluorescence

Immunofluorescence was performed as described in [51] with some minor modifications. Yeast cells were pre-grown at 30°C with agitation (200 rpm) in selective SC medium containing raffinose. After this pre-incubation, cells were re-inoculated at $OD_{600} = 0,2$ in 4 ml of selective SC medium containing galactose and incubated overnight at 30°C (W303-1A) or 37°C (BY4741 WT) with agitation (200 rpm). Cells were then fixed in 4% formaldehyde for 1 hour at 30°C. Cells were then harvested by centrifugation, washed in sterile water, treated to form spheroplasts, (1 hour at 30° in K_2HPO_4 50 mM, KH_2PO_4 50 mM, $MgCl_2$ 0,5 mM, sorbitol 1,2 M, βME 70 mM, Lyticase 50 µg/ml, pH 6,8), washed in PBS and fixed onto poly-lysine-coated slides. Permeabilization was then performed with PBS containing 0,2% Triton-X100 for 15 minutes, after which cells were washed two times with PBS and subsequently blocked with PBS containing 0,5% BSA for 10 minutes. Fixed cells were then incubated with anti-ASYN primary antibody (ASYN Purified Mouse Anti-α-Synuclein, BD) diluted 1:100 in PBS containing 0,1% BSA for 1 hour, washed and incubated with CY3 conjugated secondary antibody (Cy3® Goat Anti-Mouse IgG (H+L) Invitrogen) diluted 1:500 in PBS containing 0,1% BSA for 30 minutes. After antibody labelling samples were mounted in a glycerol mounting media containing DAPI (1 mg/ml in 90% glycerol) and analysed using a Zeiss AxioObserver.D1 microscope. Image acquisition was performed with the software AxioVision (Zeiss). Statistical significance was determined by performing one-way ANOVA with post-hoc Dunnett's test using GraphPad Prism.

Results

Cytotoxic Growth Effects Upon High-copy Plasmid Expression of ASYN Alone or in Combination with Tau

We started promoting the co-expression of ASYN and tau 2N/4R isoforms in yeast by transforming the WT strain BY4741 with a bi-directional inducible high-copy plasmid containing ASYN (WT or A53T) and tau (WT or P301L) coding sequences alone or in combination. By using a bi-directional vector, our goal was to achieve a more controlled expression of both transgenes, as the use of two independent episomal plasmids results in copy number variation and, subsequently, in different and variable levels of protein expression. As observed by western blot, all the transformants showed equal protein expression levels of ASYN isoforms, while expression of tau isoforms strongly decreased when expressed in combination with ASYN (WT or A53T) (Fig. 1,A). This decrease is not a specific effect to ASYN, as co-expression of tau isoforms with a control protein led to the same alterations in tau total protein levels (data not shown). The growth profile upon expression of ASYN and tau variants alone and in combination was evaluated in both solid and liquid media. Dot spot analysis revealed that expression of ASYN (WT or A53T) at 37°C causes growth delay, as reported in previous studies [40]. In contrast, expression of tau (WT or P301L) had no effect on yeast fitness which has also been observed previously [38]. Yeast strains co-expressing ASYN isoforms and tau showed a similar growth delay as observed upon expression of ASYN alone, with no evident synergistic effect being detected. In contrast to the cytotoxic growth effect observed for ASYN (WT or A53T) expressed alone or in combination with tau (WT or P301L) in solid media, no growth delay was observed in liquid media (Fig. 1,C). These data strongly suggest that the cytotoxic growth effect phenotype observed in these episomal strains is mainly mediated by the expression of ASYN isoforms. Nevertheless, tau levels were

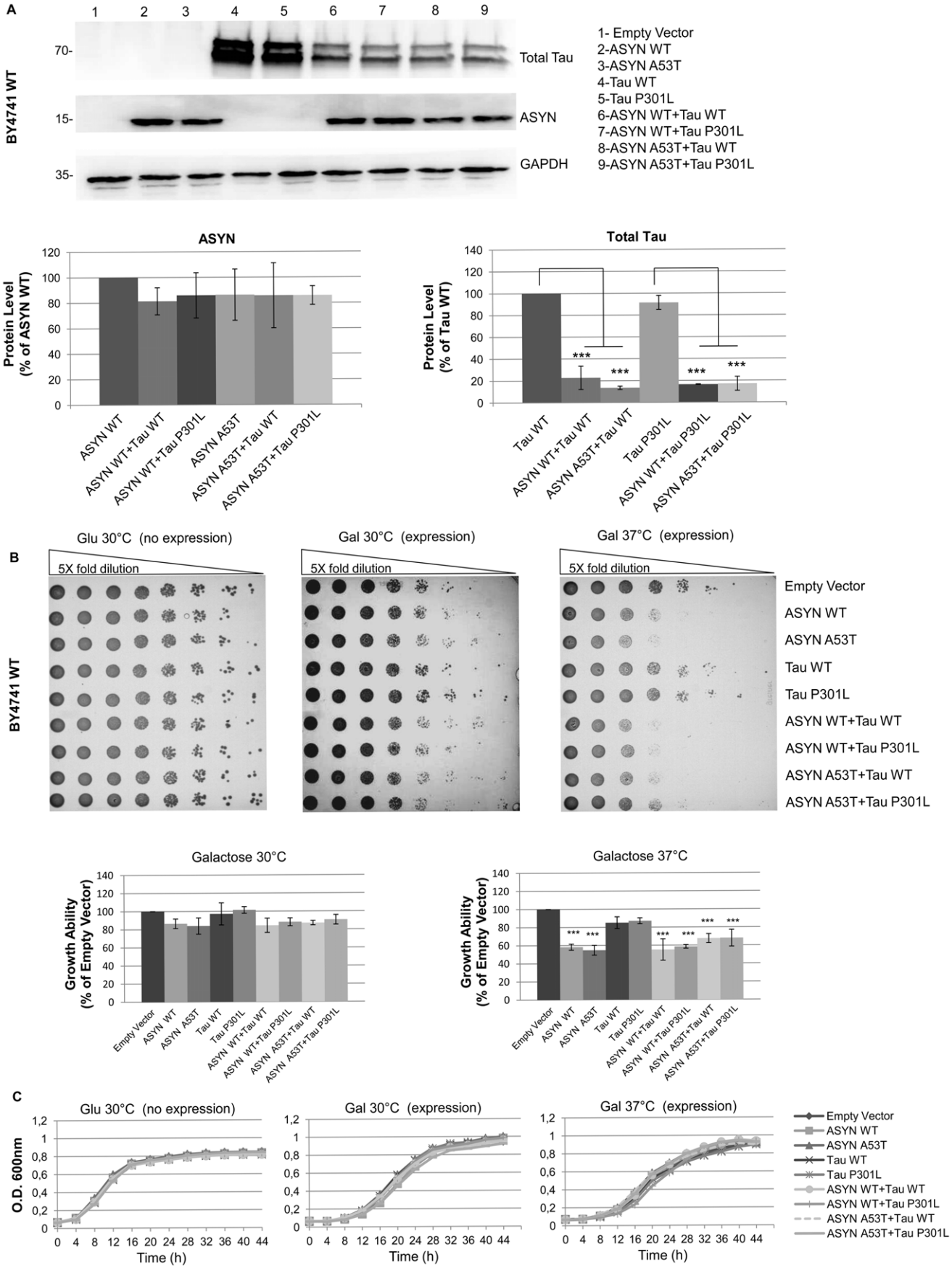
A
1 2 3 4 5 6 7 8 9
BY4741 WT
70-
15-
35-
Total Tau
ASYN
GAPDH
1- Empty Vector
2-ASYN WT
3-ASYN A53T
4-Tau WT
5-Tau P301L
6-ASYN WT+Tau WT
7-ASYN WT+Tau P301L
8-ASYN A53T+Tau WT
9-ASYN A53T+Tau P301L
ASYN
Protein Level (% of ASYN WT)
ASYN WT
ASYN WT+Tau WT
ASYN WT+Tau P301L
ASYN A53T
ASYN A53T+Tau WT
ASYN A53T+Tau P301L
Total Tau
Protein Level (% of Tau WT)
Tau WT
ASYN WT+Tau WT
ASYN A53T+Tau WT
Tau P301L
ASYN WT+Tau P301L
ASYN A53T+Tau P301L
***
B
Glu 30°C (no expression)
Gal 30°C (expression)
Gal 37°C (expression)
5X fold dilution
BY4741 WT
Empty Vector
ASYN WT
ASYN A53T
Tau WT
Tau P301L
ASYN WT+Tau WT
ASYN WT+Tau P301L
ASYN A53T+Tau WT
ASYN A53T+Tau P301L
Galactose 30°C
Galactose 37°C
Growth Ability (% of Empty Vector)
C
Glu 30°C (no expression)
Gal 30°C (expression)
Gal 37°C (expression)
O.D. 600nm
Time (h)
Empty Vector
ASYN WT
ASYN A53T
Tau WT
Tau P301L
ASYN WT+Tau WT
ASYN WT+Tau P301L
ASYN A53T+Tau WT
ASYN A53T+Tau P301L

Figure 1. Cytotoxic growth effects upon high-copy plasmid expression of ASYN alone or in combination with Tau. A) High expression level of ASYN (WT and A53T) and tau (WT and P301L), detectable by western blot upon expression with pESC-LEU bidirectional high-copy (2μ) episomal vector. Whereas ASYN levels (WT and A53T) are similar when either expressed alone or in combination with tau (WT or P301L), tau (WT and P301L) levels are strongly decreased when expressed in combination with ASYN (WT or A53T), (***p<0,001). GAPDH was used as loading control. B) Strong growth delay observed by dot spot at 37°C after 72 hours of incubation upon expression of ASYN (WT and A53T) alone or in combination with tau (WT and P301L). Expression of tau (WT or P301L) alone has no effect on cells vitality whereas when expressed in combination with ASYN (WT and A53T) cytotoxicity is observed. A quantitative plot of the dot spots is shown. Results are representative of at least three independent experiments. C) No differences observed upon expression of ASYN and tau isoforms alone or in combination by growth ability tests performed in liquid media using 96 well plates at both 30°C and 37°C. All results are representative of at least three independent experiments.
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reduced upon co-expression, which might have hindered the observation of a synergistic phenotype.

ASYN Increases Tau Insoluble Aggregation State

It is demonstrated that in mammalian cellular models tau co-localizes with ASYN aggregates with overexpression of tau leading to an increase in the number of ASYN inclusions and to a reduction in their size [31,52]. To further characterize and to assess if similar effects are present in the episomal yeast strains generated, the percentage of cells showing ASYN intracellular inclusions was quantified by immunofluorescence. No differences were observed, in terms of size and number of ASYN intracellular inclusions between the strains expressing ASYN isoforms alone or in combination with tau isoforms (Fig. 2,A). Interestingly, in the double mutant ASYN A53T/tau P301L, cells with large aggregates were not detected (Fig. 2,A). In order to quantify tau intracellular inclusions, anti-tau immunofluorescence was also tried using different antibodies but no specific signal was ever detected. As an alternative, sarkosyl fractionation was applied as this technique is largely used to characterize the solubility of intracellular protein aggregates in the brain of PD or AD patients [53,54] as well as in animal and cellular models of neurodegeneration [43,55,56]. Results obtained showed that all ASYN and tau isoforms, expressed alone or in combination, are present in the sarkosyl insoluble fraction (Fig. 2,B). ASYN isoforms alone or in combination appear to be evenly distributed between the soluble and insoluble fractions. Tau WT seems to be slightly more concentrated in the insoluble fraction whereas tau P301L looks to be marginally more abundant in the soluble fraction (although this might be due to small differences in total protein loading, as GAPDH intensity also seems to be higher for this sample). Interestingly, co-expression of tau isoforms with ASYN leads to an increase in the proportion of insoluble tau (Fig. 2,B), as observed by the higher amount of tau present in the sarkosyl insoluble fraction when compared to the soluble fraction. Therefore, whereas tau expression, at least up to this protein level, does not seem to affect ASYN intracellular inclusion number and size, ASYN expression increases the insoluble fraction of tau.

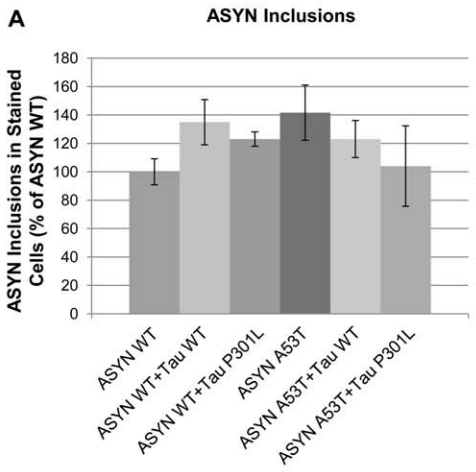
ASYN Increases Tau Phosphorylation in S396/404 via RIM11

Hyperphosphorylated tau is the main component of PHF of NFT in the brain of AD patients [57,58]. As we observed that the presence of ASYN leads to an increase in tau insoluble aggregation state (Fig. 2,B) we next evaluated the phosphorylation status of tau in the episomal yeast strain co-expressing ASYN and tau. To assess tau phosphorylation level, western blot analysis was performed using AD2 antibody, which recognizes phosphorylated tau at S396/404. This form of tau is crucial for tau fibrillization and [18,19] is characteristic of PHF in Alzheimer's disease [59]. Analysis of AD2 immunoreactivity versus total tau showed that tau WT and tau P301L are phosphorylated to the same extend when expressed alone (Fig. 3,A) but display a significant increase in S396/404 phosphorylation in the presence of ASYN. The increased relative level of phosphorylation of tau isoforms is even more striking upon co-expression with ASYN A53T (Fig. 3,A).

S306/404 of tau is a typical *GSK3β* substrate [20,42]. In order to evaluate if the increased tau phosphorylation observed upon co-expression of ASYN was mediated by *GSK3β*, we used a yeast strain lacking the orthologue of human *GSK3β*. Yeast cells carry four orthologues of human *GSK3β* and it is already known that *RIM11* can act as a tau kinase and that the AD2 phosphoepitope is significantly reduced or absent in these mutant strains [42,60]. Hence, the inducible high-copy plasmid expressing ASYN (WT or A53T) and tau (WT or P301L), alone or in combination, was transformed in a ΔRIM11 mutant yeast strain, in order to evaluate tau phosphorylation state in the presence and absence of ASYN. Results showed that the AD2 tau phosphoepitope was not detected in any of the samples tested, corroborating that the increased tau phosphorylation in S306/404 observed in the presence of ASYN is fully mediated by *RIM11*. Growth ability test in solid media showed that lacking of tau phosphorylation in S396/404 does not alter the growth phenotype observed in BY4741 WT yeast strain (Fig. 3,C). This was an expected outcome, as expression of tau seems to be non-related to the growth arrest effect observed. Taken together, the finding that tau phosphorylation in S396/404 via *RIM11* is increased in the presence of ASYN is in accordance with what has been described in other models, where it was demonstrated that *in vitro* ASYN directly stimulates tau phosphorylation via *GSK3β* [61], while *in vivo* and in post-mortem brain from PD high levels of active *GSK3β* and hyperphosphorylated tau were observed [62,63]. Our yeast strain thus mimics relevant pathological features of neurodegeneration diseases where interaction between ASYN and tau occurs. Nevertheless, increased cytotoxicity due to tau hyperphosphorylation was not observed. This might be due to the lower tau intracellular levels upon co-expression with ASYN. A model where tau protein levels were higher would facilitate the assessment of pathological synergistic interactions with ASYN.

Strong Synergistic Growth Effect Upon Genome Integration of ASYN WT and Tau WT

We next accessed the synergistic phenotype of ASYN and tau expression using an integrative yeast model by inserting one copy of ASYN (WT or A53T) and tau (WT or P301L), alone or in combination, into the genome of W303-1A yeast strain. Our goal was to use a more stable and independent system for the expression of the two transgenes, in order to achieve higher intracellular tau levels. Integration was performed using specific yeast vectors able to recombine with the yeast genomic DNA at defined sites and correct integration was confirmed by PCR. After generation of the integrative strains, ASYN and tau protein expression level and tau phosphorylation in S396/404 were evaluated by western blot. As desired, tau (WT and P301L) expression levels, alone and in combination with ASYN, were significantly higher and constant among all strains (Fig. 4,A). ASYN protein levels were generally constant, with



Strains	ASYN Inclusions in Stained Cells (%)			
	Small inclusions		Big inclusions	
	mean	±sd	mean	±sd
ASYN WT	92,3	10,8	7,7	3,9
ASYN WT+Tau WT	129,8	37,7	15,1	7,8
ASYN WT+Tau P301L	102,7	14,5	14,6	8,1
ASYN A53T	122,2	11,6	19,4	8,1
ASYN A53T+Tau WT	113,4	10,1	12,2	6,1
ASYN A53T+Tau P301L	121,1	6,5	0	0

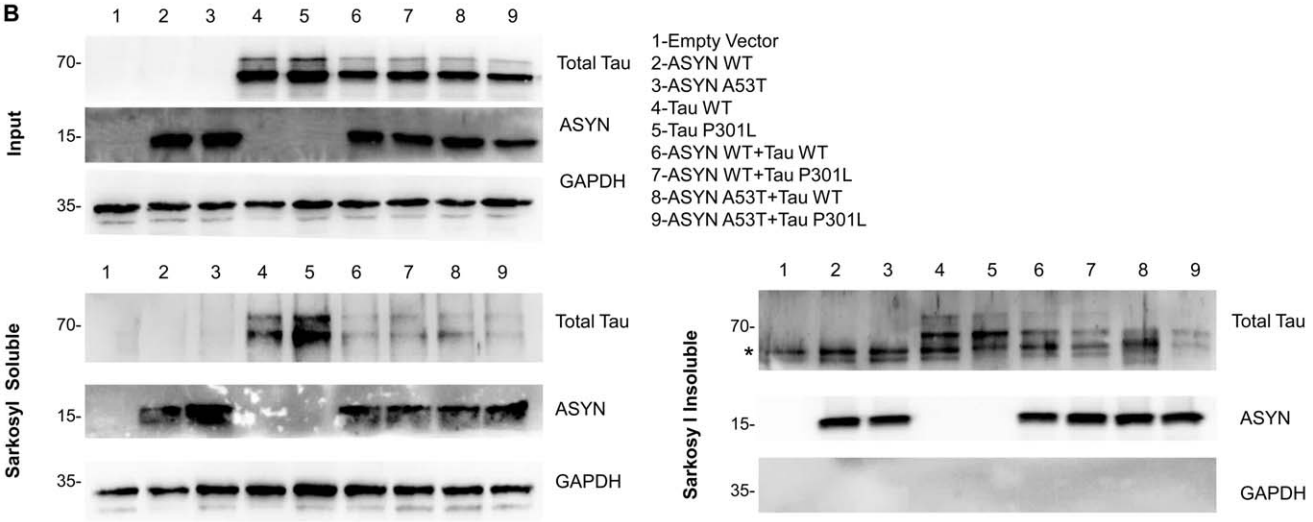


Figure 2. ASYN increases Tau insoluble aggregation state. A) Immunofluorescence with an anti-ASYN antibody showed no significant differences between the percentage of cells that contain ASYN (WT and A53T) intracellular inclusions when expressed alone or in combination with tau (WT and P301L). No yeast cells with ASYN big inclusions were observed in the strain expressing ASYN A53T in combination with tau P301L For statistical analysis at least 800 cells were counted. B) Both ASYN (WT and A53T) and tau (WT and P301L) form intracellular sarkosyl insoluble aggregates when expressed either alone or in combination, which are detectable by western blot. GAPDH was used as loading and soluble protein control. *corresponds to an unspecific band. Results are representative of three independent experiments.
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the exception of strains co-expressing tau P301L, which displayed reduced expression of ASYN, for both WT and A53T isoforms (Fig. 4,A). The observed differences in ASYN protein levels can not be interpreted as a specific consequence of tau co-expression, as they might merely reflect clonal variation among strains. Interpretation of these differences needs further experimental clarification. Quantification of tau phosphorylation levels revealed that the presence of ASYN did not induce significant changes, most probably due to the lower levels of intracellular ASYN (or, at least, to the lower ratio of ASYN/tau) (Fig. 4,A). Exception to this was the strain co-expressing tau WT and ASYN WT, which displayed a huge increase in the relative fraction of phosphorylated tau.

Cytotoxic growth effects were also assessed in this integrative model in both solid and liquid media. As expected, dot spot results showed that expression of ASYN WT and A53T at 30°C caused moderate and high cytotoxic growth phenotypes, respectively (Fig. 4,B). Despite the high expression level of tau isoforms in this integrative model, tau expression alone didn't show any effect in yeast growth in either solid or liquid media (Fig. 4,B and C). A strong synergistic growth effect was observed upon co-expression of ASYN WT and tau WT (Fig.4,B), which was even more pronounced at 37°C (Fig. 4,B). A similar growth delay was also obtained in liquid media showing the robustness of the alterations observed (Fig. 4,C). The synergistic growth effect observed for this integrative strain seems to be correlated with the increased tau phosphorylation state.

We observed an apparent rescue of toxic phenotype upon co-expression of ASYN (WT and A53T) with tau P301L, as the corresponding strains display increased growth fitness (Fig. 4, B). Nevertheless, expression levels of ASYN WT and ASYN A53T in both these strains is significantly lower (Fig. 4, A), and thus the improved growth might be merely a consequence of a less toxic intracellular environment due to less ASYN and not a true rescue by P301L.

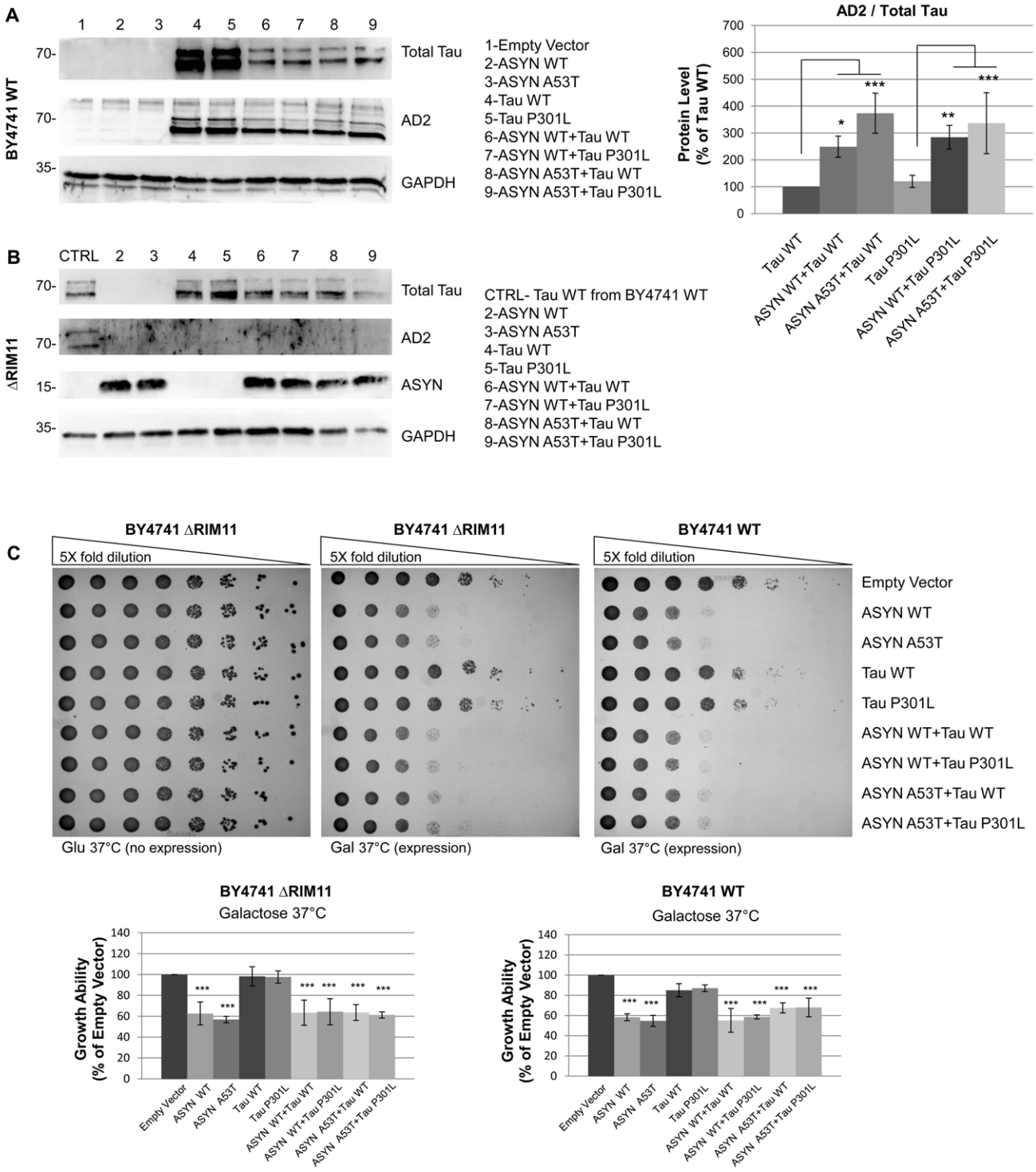

Figure 3. ASYN increases Tau phosphorylation in S396/404 via *RIM11*. A) Relative percentage of phosphorylated tau (WT and P301L) in S396/404 labelled by the antibody AD2 was increased in the presence of ASYN (WT and A53T). (*p<0,05; **p<0,01). GAPDH was used as loading control. B) No phosphorylated tau (WT or P301L) detected in ΔRIM11 mutant by western blot when expressed alone or in combination with ASYN (WT or A53T). GAPDH was used as loading control. C) Lack of tau (WT and P301L) phosphorylation in S396/404 doesn't alter cytotoxicity observed in solid media when tau (WT or P301L) and ASYN (WT and A53T) are expressed alone or in combination in ΔRIM11 as compared to expression in BY4741 WT.A quantitative plot of the dot spots is shown (*p<0,05; **p<0,01; ***p<0,001). Results are representative of at least three independent experiments. doi:10.1371/journal.pone.0055848.g003

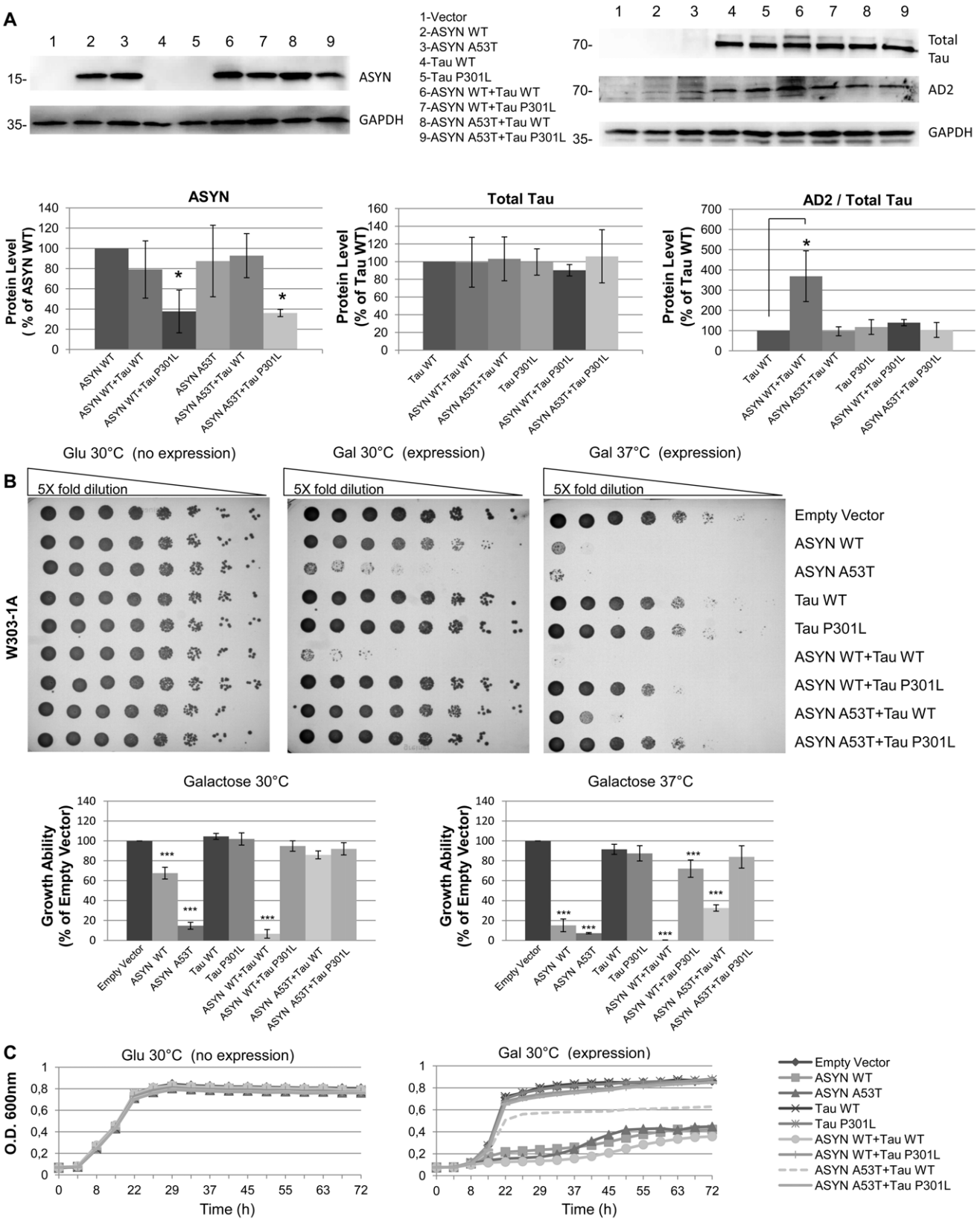

Figure 4. Strong synergistic growth effect upon genome integration of ASYN WT and Tau WT. A) ASYN protein expression levels detectable by western blot, remain generally constant, displaying a reduction upon co-expression of either tau isoform. Total tau expression levels remain constant whereas the percentage of tau WT phosphorylated in S396/404 increases only in the presence of ASYN WT. (*p<0,05; **p<0,01). GAPDH was used as loading control. B) Slight growth delay observed by dot spot at 30°C upon expression of ASYN WT, high growth delay with ASYN A53T and strong synergistic cytotoxicity observed when ASYN WT and tau WT are co-expressed. At 37°C a total growth arrest is observed for the

strains expressing either ASYN (WT or A53T) alone or expressing ASYN WT in combination with tau WT. Tau P301L seems to rescue cytotoxic effect mediated by ASYN (WT and A53T) as well as tau WT when in combination with ASYN A53T. A quantitative plot of the dot spots is shown (*p<0,05; ***p<0,001). Results are representative of at least three independent experiments. C) Strong growth delay also observed in liquid growth analysis at 30°C upon co-expression of ASYN (WT and A53T) alone or ASYN WT in combination with tau WT. As in solid media tau P301L seems to rescue cytotoxic effect mediated by ASYN (WT and A53T) as well as tau WT when in combination with ASYN A53T. All results are representative of at least three independent experiments.
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High Percentage of ASYN Cytoplasmatic Inclusions and Insoluble Tau Correlate with the Synergistic Growth Effect

To further investigate the synergistic interaction detected between ASYN and tau in the integrative yeast strains developed in this work, we accessed the aggregation state of ASYN and tau by immunofluorescence and sarkosyl fractionation experiments. Interestingly, the number of ASYN intracellular inclusions detected by immunofluorescence didn't remain constant in all strains. Strains expressing ASYN alone or ASYN WT in combination with tau WT displayed a high level of cells with ASYN inclusions. A reduction in the number of cells with inclusions was observed in the strain expressing ASYN WT in combination with tau P301L and in both the strains expressing ASYN A53T in combination with tau (WT or P301L) (Fig. 5,A). Furthermore, changes in ASYN intracellular inclusion localization were also observed. It is known that in yeast ASYN initially localizes to the membrane, and subsequently starts to form small membrane localized inclusions that finally convert into larger cytoplasmatic inclusions [44]. The majority of yeast cells expressing ASYN isoforms alone and ASYN WT in combination with tau WT showed only the most advanced cytoplasmatic inclusions. On the other hand, the strain co-expressing ASYN WT and tau P301L showed only small membrane connected inclusions, whereas in the strains co-expressing ASYN A53T and tau isoforms both membrane connected and cytoplasmatic inclusions were observed (Fig. 5,B). Sarkosyl fractionation assays showed ASYN equally distributed between the sarkosyl soluble and insoluble fractions in all the strains expressing ASYN isoforms alone or in combination with tau isoforms, whereas insoluble tau WT was only detected when co-expressed with ASYN WT (Fig. 5,C). These findings suggest that the synergistic growth effect observed upon co-expression of ASYN WT and tau WT in this integrative model is probably triggered by the high percentage of ASYN cytoplasmatic inclusions and by the increased fraction of insoluble tau.

Discussion

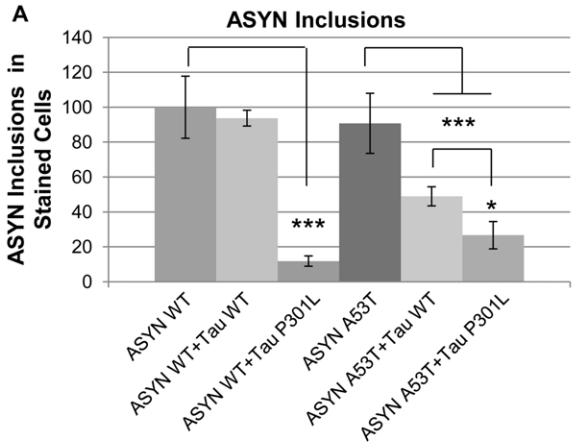
Interactions between ASYN and tau have been shown to occur in several neurodegenerative diseases, as relevant as AD and PD. Although some light has been shed on the cellular effects resulting from this interaction [64], a full understanding of the cellular mechanisms triggered by this toxic duet has not been achieved yet. In this work two different yeast sets of strains were developed and characterized with the final aim of being used as tools for the discovery of potential target genes that mediate the interaction between ASYN and tau.

We started by promoting the expression of ASYN and tau isoforms in yeast from a bidirectional episomal vector. Expression of ASYN isoforms alone led to a cytotoxic growth phenotype, as reported before [40]. On the contrary, no changes in yeast growth properties were observed upon expression of tau WT or mutants, also in accordance with previous observations [43]. In our study, co-expression of ASYN and tau from a bidirectional episomal vector resulted in no evident synergistic toxic effect. Previous data showed that co-expression of tau and ASYN in yeast resulted in synergistic growth delay [44], although in that case, no cytotoxicity upon expression of ASYN alone was observed, in clear contrast to the majority of the published studies.

In our episomal co-expression strains, ASYN levels remained constant in all the strains analysed, whereas tau levels strongly decreased upon co-expression with ASYN. The observed reduction in tau protein levels is not specific to the co-expression of ASYN. Similar results were observed upon co-expression of tau and other proteins and most probably reflect a lower protein expression efficiency resulting from the simultaneous use of the Gal1-Gal10 divergent promoter and subsequent downstream processes. ASYN levels were not affected by co-expression of a second transgene, which might be due to the small size of this transcript, which allows higher protein expression efficiency. Overall, co-expression of ASYN and tau had no evident effect on the size and number of ASYN intracellular inclusions.

Our results showed that the presence of ASYN affected the solubility of tau, increasing the fraction of insoluble/aggregated protein, both for tau WT and tau P301L. To further explore this evidence we analysed tau phosphorylation at Ser396/404, as tau phosphorylation is known to lead to the formation of insoluble tau aggregates. Indeed, co-expression of ASYN isoforms with either tau WT or tau P301L led to a significant increase in tau phosphorylation, particularly in the case of co-expression of tau isoforms with ASYN A53T. ASYN has been reported to directly stimulate tau phosphorylation by *GSK3β* by making part of a heterotrimeric complex containing ASYN, tau and *GSK3β* [30,61]. The pathological epitope Ser396/404 is a typical *GSK3β* substrate, already shown to be a target for tau phosphorylation in yeast [42]. We showed that in our model the presence of ASYN led to increased aggregation of tau through phosphorylation at Ser396/404 via *RIM11*, the yeast orthologue of *GSK3β*. In RIM11-deleted strain, no phosphorylation at Ser396/404 was observed. To our knowledge, this is the first evidence that ASYN is able to induce tau phosphorylation and aggregation in yeast, proving that yeast recapitulates the reported mechanism through which ASYN stimulates *GSK3β*, leading to phosphorylation of tau at pathogenic epitopes [61].

Despite the increased phosphorylation and aggregation of tau WT and tau P301L promoted by co-expression of ASYN isoforms, no synergistic differences in growth phenotype were detected due to alterations in phosphorylated tau in our episomal yeast strain. Nevertheless, previous studies in yeast have consistently revealed that tau expression *per se* does not affect growth, despite tau phosphorylation, conformational changes and accumulation into aggregates [38]. In addition, the precise nature of the toxic tau species and the exact sequence of events leading to tau-mediated toxicity are not consensual. Several lines of evidence suggest that tau aggregates are not the main cause of cell death, as neuron loss can be observed in the absence of tau tangles in both Drosophila and mice models [65–67] and other mice models show dissociation between brain areas of neurofibrillary tangles formation and neuronal loss [68,69]. Results obtained with our episomal strain therefore mimic a relevant feature of human neurodegeneration, although not resulting in a measurable synergistic phenotype of



B

ASYN Inclusions State After 16h

Strains	ASYN Stained Cells (%)					
	Stage 1 Membrane		Stage 2 Membrane and Cytoplasmatic		Stage 3 Cytoplasmatic	
	mean	±SD	mean	±SD	mean	±SD
ASYN WT	-	-	0,4	0,2	99,6	0,2
ASYN A53T	-	-	0,2	0,4	99,8	0,4
ASYN WT+Tau WT	-	-	0,3	0,3	99,8	0,3
ASYN WT+Tau P301L	97,9	0,4	2,1	0,4	-	-
ASYN A53T+Tau WT	7,7	2,1	92,3	2,1	-	-
ASYN A53T+Tau P301L	2,6	1,4	97,4	1,4	-	-

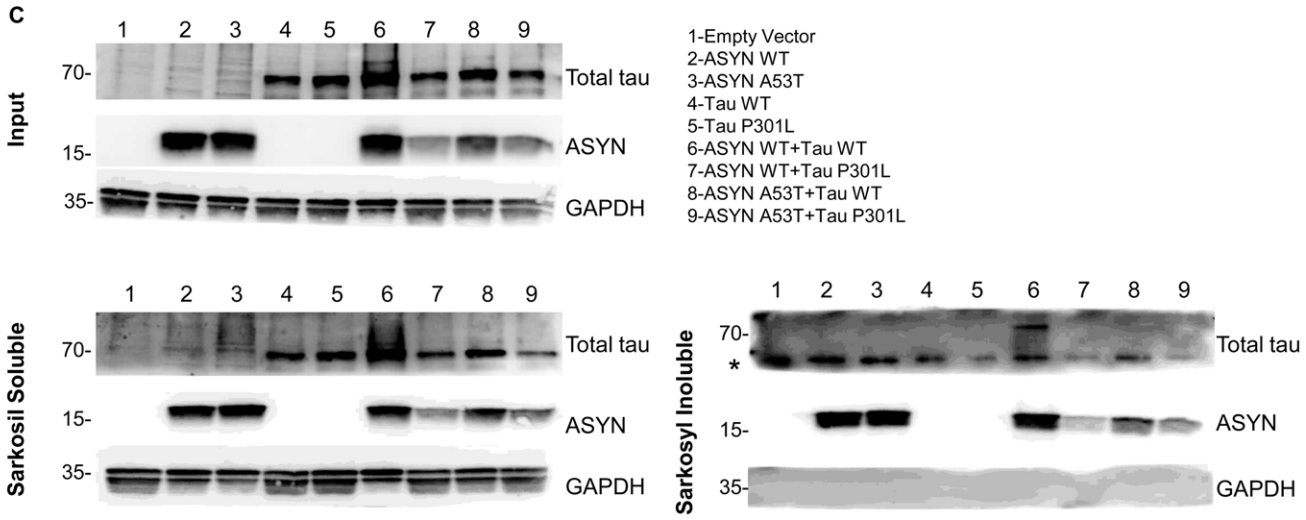


Figure 5. High percentage of ASYN cytoplasmatic inclusions and insoluble Tau correlate with the observed synergistic growth effect. A) Percentage of cells showing ASYN intracellular aggregates, detectable by immunofluorescence with anti-ASYN antibody, is similar when ASYN WT is expressed alone or in combination with tau WT, while when it is in combination with tau P301L it is highly reduced. Co-expression of ASYN A53T with tau (WT or P301L) also shows a decreased percentage of cells with intracellular aggregates. (*p<0,05; ***p<0,001). For statistical

analysis at least 800 cells were counted. B) ASYN inclusions localization analyzed after 16 h, by immunofluorescence with anti-ASYN antibody, shows that in the strain expressing ASYN (WT or A53T) alone or ASYN WT in combination with tau WT inclusions are mainly cytoplasmatic. In the strains co-expressing ASYN A53T in combination with tau (WT or P301L) both small membrane associated and cytoplasmatic inclusions are detectable, whereas only small membrane associated inclusions can be observed when ASYN WT is expressed in combination with tau P301L For statistical analysis at least 800 cells were counted. C) ASYN (WT and A53T) alone or in combination with tau (WT or P301L) is detectable by western blot in the sarkosyl insoluble fraction, whereas tau is detectable in the sarkosyl insoluble fraction only when co-expressed with ASYN WT. GAPDH was used as loading and soluble protein control. *corresponds to an unspecific band. Results are representative of at least three independent experiments.
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growth arrest. Different observations were previously described for ASYN and tau co-expression in yeast [44]. Nevertheless, the authors used a different approach, expressing each protein from an independent episomal plasmid (and thus not allowing for copy number control and stability). Additionally, tau protein levels and phosphorylation state were not accessed in the reported study, thus not allowing a more direct comparison with the results obtained in the present work.

We next evaluated the co-expression of ASYN and tau isoforms in yeast by making use of a different experimental approach, based on the stable integration of one copy of each transgene in the yeast genome. Our main goal was to achieve higher tau expression levels, in order to maximize any eventual underlying phenotypic change. Using this integrative approach, tau protein levels were found to be constant in all the generated strains, whereas ASYN levels showed variations. Again, expression of ASYN isoforms alone caused cytotoxic growth delay, more pronounced in the case of ASYN A53T. Despite the high and constant expression levels, the presence of tau isoforms alone had no implication in yeast growth phenotype.

Yeast strains co-expressing tau P301L with either ASYN isoform seemed to display a milder phenotype than strains expressing ASYN isoforms alone. Nevertheless, this "rescue" is only apparent has it reflects the low levels of ASYN in the yeast strains co-expressing tau P301L. Accordingly, strains co-expressing ASYN isoforms with tau P301L display a reduced number of ASYN inclusions when compared to the strains expressing ASYN WT or A53T alone. Moreover, these inclusions were predominantly located at the cell membrane, indicating an early stage of toxicity [40], as no cytoplasmatic ASYN aggregates were observed.

A strong synergistic effect in yeast growth was observed upon stable integration and co-expression of ASYN WT and tau WT. Similar observations were previously reported for the co-expression of these two proteins from two episomal vectors in yeast [44], although the typical growth delay upon expression of ASYN alone did not occur. The synergistic phenotype observed in our study was associated with increased phosphorylation of tau at Ser396/404, presumably via *RIM11* as a consequence of stimulation by ASYN. High levels of ASYN intracellular inclusions were present in this strain, all located in the cytoplasm and thus representative of late aggregation stages [40]. Most importantly, the yeast strain expressing ASYN WT and tau WT also showed to display an increased fraction of insoluble tau. It is known that ASYN toxicity in yeast is directly correlated to aggregation and inclusion formation [40,70] and the number of cells which displayed ASYN inclusions, as well as the localization of those inclusions, were identical between the strain expressing ASYN WT and the strain co-expressing ASYN WT and tau WT. As no differences in the pattern of cytoplasmatic inclusions were registered, the increased toxic phenotype observed must be triggered by the high intracellular levels of both proteins, leading to the concomitant presence of a high number ASYN inclusions and a high amount of phosphorylated and aggregated tau. Occurrence of a measurable synergistic growth arrest phenotype was also favoured by the use of the W303-1A yeast strain, whose genetic background is more sensitive than the BY4741 yeast strain. With W303-1A, evident growth delay upon expression of ASYN alone can be observed already at 30°C (Fig. 4,B) whereas for BY4741 only at 37°C the same phenotype is achieved (Fig. 1, B). Three mutations differentiate W303-1A from BY4741: 1) Ybp1-1 mutation which abolishes *Ybp1* function, increasing sensitivity to oxidative stress, 2) Rad5-G535R missense mutation which abolishes *Rad5* function, involved in DNA repair and 3) *Bud4* affecting axial budding [71–73]. The use of a genetically more sensitive strain, together with the high levels of ASYN inclusion formation and tau phosphorylation and aggregation favoured the occurrence of a measurable synergistic cytotoxic phenotype [74].

In summary, our findings demonstrated that for the yeast strains developed, ASYN directly induced tau phosphorylation in S396/404 via *RIM11* and that formation of tau insoluble aggregates seemed to be dependent on tau phosphorylation level. Expression of ASYN, but not tau alone, led to growth retardation and a strong synergistic growth effect was observed upon genome integration of ASYN WT and tau WT. Therefore, we have developed yeast strains that recapitulate many of the most relevant aspects of ASYN and tau interactions in human pathology. The choice of yeast as the model organism will allow easy genetic manipulation for the identification of genes that can modulate the interaction between ASYN and tau. The episomal and integrative yeast strains presented here are powerful tools for rapid genome-wide analysis, allowing the combined development of both yeast knockout and plasmid overexpression screenings. In particular, the integrative strain displaying a synergistic toxic phenotype due to the co-expression of ASYN and tau is the model of choice for conducting screens in the search for genes that can be future targets for the development of new therapies for neurodegenerative diseases.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: GC AM HV PC. Performed the experiments: GC CR. Analyzed the data: GC AM PC HV. Wrote the paper: GC AM PC.

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Yeast Models Supplementary Data

Unpublished Results

Tau Phosphorylation at S396/404 Mediates the Synergistic Cytotoxic Effect

It is known that tau hyperphosphorylation is an early event leading to the formation of NFTs in which hyperphosphorylation at S396/404 is a pathological feature [472]. We previously showed that the AD2 tau phospho-epitope was not detected in the Δ*RIM11* mutant yeast strain, transformed with the inducible high-copy plasmid expressing tau (WT or P301L) alone or in combination with ASYN (WT or A53T) [501]. Thus, to further assess the role of tau phosphorylation at S396/404 in the synergistic cytotoxic effect observed, the *RIM11* gene was removed from the integrative yeast strains expressing ASYN WT and tau WT alone or in combination.

The single deletion mutants W303-1A were obtained upon *RIM11* replacement with the *HIS3* gene amplified from p413 GAL1 (ATCC-87326) using the following primers: Fw_atgaatattcaaagcaataattctccgaatctcagtaataacaaattcccgtttt aagagcttg and Rw_ttacttagacttcggatatagctttttttttacagatgatctacataagaacacctttggtgg. W303-1A yeast strains were then transformed with the *HIS3* PCR product. Both transformation of the yeast strains using the lithium acetate method and transformants colony selection were performed as described in [502]. Confirmation of *HIS3* gene integration was performed by PCR using the following primers: Fw_cattacaacacggcaacact and Rw_aaagaaaattgcgggaaagg. As expected, western blot analysis revealed that tau phosphorylation at S396/404 is strongly reduced if not absent upon removal of *RIM11* gene (Figure 23,A). Most importantly, growth ability test in solid media showed that absence of tau phosphorylation at S396/404 abrogates the synergistic phenotype obtained (Figure 23,B), proving that the enhanced cytotoxicity is really mediated by the cooperation between ASYN WT and tau WT. These results suggest that ASYN mediated hyperphosphorylation of tau at S396/404 might give rise to cytotoxic oligomeric tau species, since this synergistic cytotoxicity

is only observed in the yeast strain showing the higher level of AD2 phospho tau in correlation with the presence of tau in the sarkosyl insoluble fraction. Recapitulating, in our integrative yeast model ASYN WT indirectly stimulates tau WT phosphorylation at S396/404 via Rim11, promoting its fibrillization and enhancing the ASYN mediated cytotoxic effect.

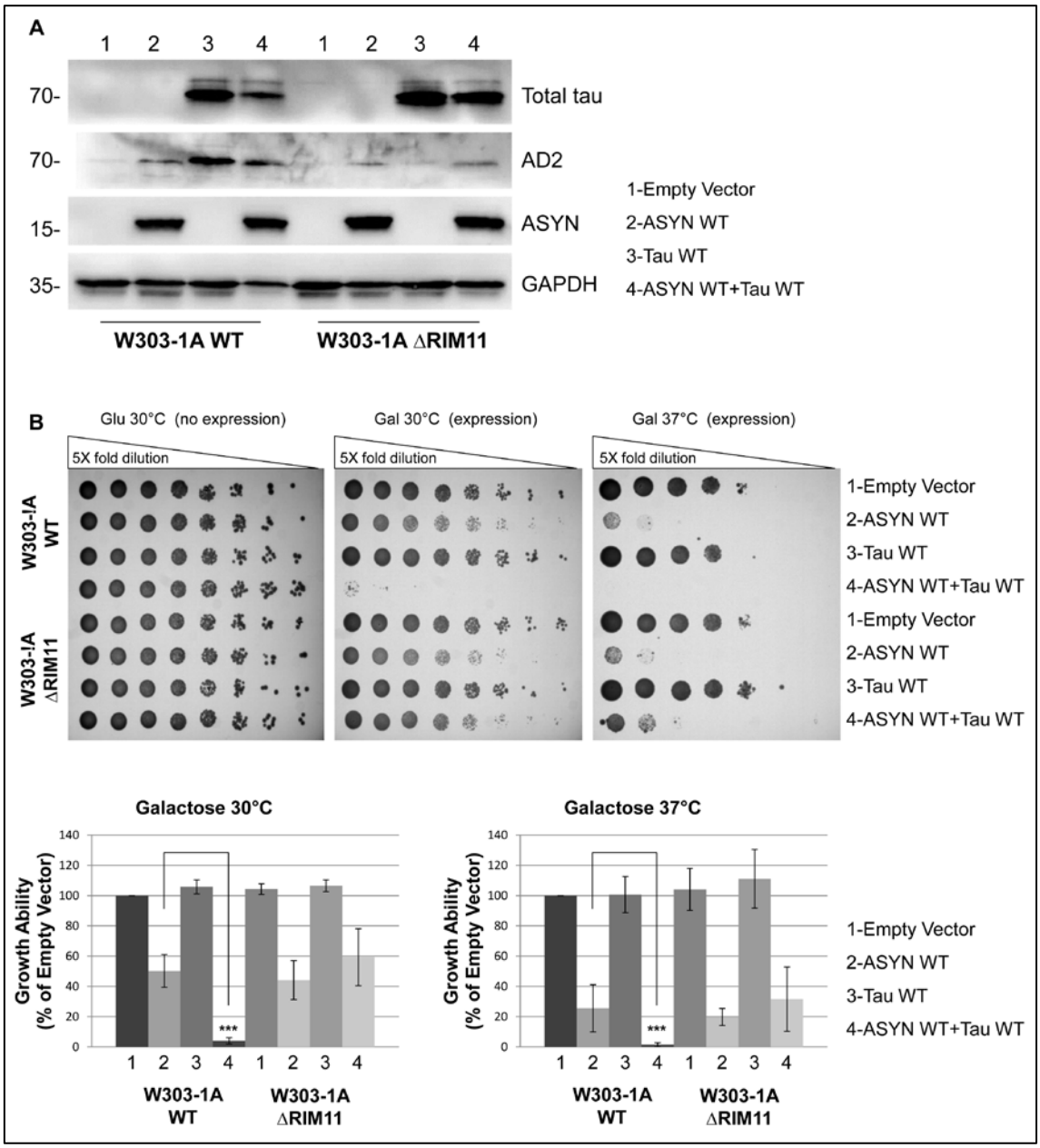

Figure 23: Tau phosphorylation at S396/404 mediates the synergistic cytotoxic effect. A) Strongly reduction or absence of phosphorylated tau WT detected in Δ*RIM11* mutants by western blot when expressed alone or in combination with ASYN WT. GAPDH was used as loading control. **B)** Lack of tau phosphorylation at S396/404 rescues the synergistic cytotoxicity observed in solid media when tau WT and ASYN WT are expressed in combination in Δ*RIM11* as compared to expression in WT yeast strain. A quantitative plot of the dot spots is shown (***p<0,001). Results are representative of at least three independent experiments.

2. Identification of Modulators of ASYN and Tau Synergistic Toxicity by GWS and HTS Assays

Unpublished Results

Identification of Modulators of ASYN and Tau Synergistic Toxicity by GWS and HTS Assays

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Abstract

Several reports have proven a consistent overlap between synucleinopathies and tauopathies suggesting that a synergistic neurotoxic effect might exist. Alpha-Synuclein (ASYN) was found in neurofibrillary tangles mainly composed by tau protein and tau was also found in Lewy bodies mainly composed by ASYN protein. Direct binding between these two proteins has been described and it was also confirmed that ASYN can act as a pathological initiator of tau phosphorylation and aggregation. We previously designed and characterized a yeast model in which the concomitant expression of the human ASYN and tau proteins is synergistically cytotoxic. The model has been used to perform high throughput screening assays (HTS) and genome wide screening assays (GWS) to identify compounds and target genes that modulate this interaction, in order to shed light into the pathological mechanism of cell dysfunction and to provide new strategies for future therapeutic intervention. By GWS and HTS preliminary screenings we have identified 5 different yeast genomic fragments containing a total number of 25 genes and 11 natural extracts able to interfere with the ASYN and tau mediated synergistic cytotoxicity. Both genes and extracts identified have the potential to uncover new molecular mechanisms that might be targeted for the treatment of patients affected by neurodegeneration.

Introduction

Synucleinopathies and tauopathies regroup a wide number of neurodegenerative disorders in which the presence of insoluble proteinacious aggregates generates disease-specific histopathologic lesions [1-3]. In tauopathies, one of the major pathological hallmarks corresponds to the presence of intracellular protein aggregates called neurofibrillary tangles (NFTs). NFTs are composed of paired helical filaments (PHF), which comprise pathological filamentous aggregations of abnormally hyperphosphorylated tau [4-9]. Similarly, in synucleinopathies the intraneuronal proteinacious cytoplasmic inclusions termed Lewy bodies (LB) are mainly composed by the presynaptic ASYN protein [10]. In several reports consistent overlapping between synucleinopathies and tauopathies has been reported suggesting that a synergistic neurotoxic effect might exist. Notably, direct binding between these two proteins was proved, ASYN was found within the NFTs and tau was found within LB. Tau phosphorylation is also promoted by ASYN enhancing the activity of GSK3B and each of these proteins, can act as seeds for the aggregation of the other [11-16]. Moreover, in a synucleinopathy mammalian model, co-expression of tau enhances ASYN aggregation and toxicity [15].

Starting around 1960, *S. cerevisiae* was introduced as an experimental system for molecular biology [17] and in 1996 yeast was the first eukaryotic organism for which the complete genomic sequence could be established [18,19]. Of the 6000 genes predicted to be encoded by its 12000-kb genome [20] about 80% are functionally characterized [21-23]. Most importantly, ~30% of human genes involved in the development of many human diseases have functional homologues in yeast [24]. Yeast cells are unicellular microorganisms that are easy to cultivate in large populations, in low-cost media and in a partially automated manner. Yeast are preferred for high-throughput screening of libraries due to advantages such as evaluation of compounds in a physiologically relevant environment and immediate negative selection of toxic compounds or those with poor membrane permeability [25].

Heterologous gene expression and genetic manipulations, such as insertion, deletion or mutations, are easy to perform [26] and several permanently updated online databases have become available (e.g. the Saccharomyces Genome Database - SGD; http://www.yeastgenome.org). Moreover, a collection of single deletion mutant strains is available for diploid cells and for non-essential genes for haploid cells [27] together with overexpression collections based on the yeast genome [28] or single inducible ORFs [26,29], providing powerful tools for genome-wide screening assays.

Despite the absence of a nervous system, yeast cells have been largely used to get insight into neurodegenerative disorders [30-32], as many of the pathways involved in pathology progression, such as mitochondrial activity, transcriptional regulation, intracellular trafficking and protein quality control are well conserved among yeast and human [33]. In many cases, the human gene encoding the neurodegeneration-causing protein has no ortholog in yeast. However, the heterologous expression of the human gene of interest has been widely used originating the so called “humanized yeast” [30,34]. In yeast, a synergistic cytotoxic effect upon co-expression of ASYN and tau has been already reported, however a detailed characterization of this interaction was missing [35]. In our previous study, a stable yeast model co-expressing one copy of the human wild-type ASYN and one copy of the human wild-type tau displayed a strong synergistic cytotoxic effect that correlates with the presence of intracellular protein aggregates composed by ASYN or tau and with increased phospho-tau levels at the pathological epitopes S396/404 [36]. Here, we used this model to perform a GWS using a “yeast genomic tiling collection” and to perform a HTS in order to identify both target genes and natural extracts able to interfere with the synergistic cytotoxic effect. We obtained a group of 25 potential target genes and 11 natural extracts able to rescue the ASYN and tau mediated synergistic cytotoxic effect.

Materials and Methods

Yeast Strains and Media

In this study was used the W303-1A integrative yeast model characterized in [36]. In the HTS assay was used the original strains whereas in the GWS assay the *LEU2* gene integrated with YIpLac128 Gal1 vector was replaced with the *HIS3* gene amplified from p413 GAL1 (ATCC-87326) using the following primers: Fw tcatgacattaacctataaaaatаggcgtatcacgaggccaattcccgttttaagagcttg and Rw_taaagtttatgtacaaatatcataaaaaaagagaatctttctacataagaacacctttggtgg. The yeast strains were then transformed with the *HIS3* PCR product. Both transformation using the lithium acetate method and transformants colony selection were performed as described in [37]. Confirmation of *HIS3* gene integration was performed by PCR using the following primers: Fw_acgaccatcacaccactgaa and Rw_tccatcaaatggtcaggtca. All strains were grown in synthetic complete media (SC) which consists of 0,67% yeast nitrogen base (Sigma-Aldrich), 0,067% yeast drop-out mix (MP Bio) and 2% (w/v) carbon source glucose, galactose or raffinose, (Sigma-Aldrich) depending on the experiments, solid media plates also contain 2% agar (BD).

Spotting Assays

Cell growth was analysed on solid selective SC medium to maintain selection for plasmids by spot assay by performing 5X fold serial dilutions of exponential growing cultures, starting at OD600 = 1,0. For this, cells were pre-grown overnight at 30°C with agitation (200 rpm) in selective SC medium, containing raffinose. After this pre-incubation, cells were re-inoculated in the same selective SC medium at OD600 of 0,2 and left to grow at 30°C with agitation (200 rpm) until an OD600 of 1,0 was reached. Spot plates containing glucose (non-inducing medium) or galactose (inducing medium) were incubated at 30°C or 37°C with images being acquired after 72 hours of incubation. Quantification analysis was performed using ImageJ [38] acquiring the intensity of all the spots in each lane from the plates containing galactose and then normalized versus the respective values obtained from the control plate containing glucose. Statistical significance was determined by performing one-way ANOVA with post-hoc Dunnett's test using GraphPad Prism.

Immunoblotting

Immunoblotting was conducted following standard procedures [39] with some minor modifications. Yeast cells were pre-grown at 30°C with agitation (200 rpm) in selective SC medium containing raffinose. After this pre-incubation, cells were inoculated at OD600 of 0,2 in selective SC medium containing galactose with or without natural extracts and incubated overnight at 30°C with agitation (200 rpm). Cells were harvested by centrifugation, washed in sterile water and pellets were re-suspended in 100 ml of 1X SDS sample buffer (60 mM Tris-HCl pH 6,8, 10% glycerol, 2% SDS, 70 mM β-ME, 1% bromophenol blue supplemented with 1X protease inhibitor cocktail (Sigma-Aldrich) and 1X PhosSTOP (Roche) phosphatase inhibitor cocktail). After re-suspension, pellets were lyzed by boiling for 5 minutes. 15 µl of each sample was run in a 12% SDS-PAGE gel and blotted onto PVDF membrane. Immunodetection was carried out using the following primary antibodies: Total tau (Polyclonal Rabbit anti-Human Tau, Dako) diluted 1:10000, ASYN (Purified Mouse Anti-a-Synuclein, BD) diluted 1:1000, ANTI_FLAG M1 (Purified Mouse Monoclonal anti-FLAG epitope, Sigma-Aldrich) diluted 1:3000 and GAPDH (Mouse Monoclonal Anti-GAPDH, Ambion) diluted 1:3000, all in in TBST containing 1% milk. The following secondary antibodies were used: Goat Anti-MouseIgG (H+L)-HRP Conjugate (BioRad) diluted 1:10000 and goat Anti-Rabbit IgG (H+L), Horseradish Peroxidase conjugate (Invitrogen) diluted 1:10000, all in TBST containing 1% milk. Membranes were revealed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and digital images acquired with Alliance 4.7 (UVITECH Cambridge). Quantification analysis was performed using ImageJ [38] and statistical significance was determined by performing one-way ANOVA with post-hoc Dunnett's test using GraphPad Prism.

High Throughput Screening Assay

The W303-1A integrative control strain (Empty Vectors) and the W303-1A integrative target strain (ASYN WT+Tau WT) were used for the screening of 3932 LUSOEXTRACT crude extracts. The extracts were re-suspended in DMSO and stored in 96-well plates at a concentration of 25 mg/ml. All liquid-handling steps were performed using a Janus Automated Workstation (PerkinElmer, Waltham, MA). Yeast cells were grown in a Liconic STX40 Automated Incubator and growth was monitored with a Victor 3V microplate reader (PerkinElmer). A validation assay was performed to confirm the growth profile of the target and control strains and asses the robustness of this platform by calculation of the Z factor which was 0,79. For the validation and screening assay, both strains were pre-grown at 30°C with agitation (200 rpm) in in selective SC medium containing raffinose. Then, cultures were diluted in selective SC medium containing glucose or galactose to OD600 of 0,05 and 96-well plates were filled with 196 µl of culture per well (control or target strains). LUSOEXTRACT Crude Set extracts were added to the wells containing the target strain at a final concentration of 0.5 mg/ml. Plates were then incubated in LiCONic STX40 for approximately 68 hours at 30°C and OD600 was read in Victor 3V microplate reader every 4 hours. Results obtained were analyzed with Microsoft Excel, the parameters average (AVER), standard deviation (STDEV) and maximum value (MAX) were used to determine adequate thresholds for hits determination. The threshold chosen for hits determination was: MAX + STDEV. Values of OD600 higher than this threshold were considered hits. Hit confirmation was performed in a secondary dose-response screening at four different concentrations: 0,125 – 0,25 – 0,5 and 0,75 mg/ml. In this secondary screening the control strain was also used for testing the extracts in the highest concentration of 0,75 mg/ml.

Genome Wide Screening Assay

The modified W303-1A integrative control strain (Empty Vectors) and the W303-1A integrative target strain (ASYN WT +Tau WT) were used to perform the GWS assay. In this screening we used the Yeast Genomic Tiling Collection Assay Ready DNA (Thermo Scientific Cat.YSC5103) which consists of Pooled DNA (1588 different clones in suspension) and Empty pGP564 (vector control in suspension). According with the product manual the control strain was transformed with Empty pGP564 whereas the target strain was transformed with the Pooled DNA. High efficiency transformation, transformants colonies selection and transformation efficiency calculation was executed as described in [40] with some minor modification. Transformation: heat shock at 42°C for 30 minutes followed by 2 hours of recovery at 30°C in liquid YPD media (Sigma-Aldrich) before transformants colonies selection. Transformants colony selection target strain: 96 hours of incubation at 35°C in solid selective SC medium containing galactose. The growth ability of target strain transformants colonies was then evaluated by spotting assay. From each target strain transformants colonies showing growth recovery was extracted the

contained plasmid (Qiagen, Plasmid Mini Kit) and then sequenced to identify the genomic fragments carried by each clone. The selected CDS of the corresponding genes identified upon sequencing were then amplified by PCR, C-terminal FLAG epitope tagged and cloned into the p413 Gal1 or GPD (ATCC-87326; 87354) using the following primers (Table 1). Both ttransformation of yeast the strains with the resultants p413 vectors and transformants colony selection were performed using the lithium acetate method as described in [37].

Table 1: Sequences of the primers used for the single CDS cloning and respective cloning sites.

Gene	Primers	Cloning Sites
FIG1	Fw_TTCTCTAGAATGGTCGCAATCTCAATGATTTG RW_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCAACTTTGGAATGCAATGTAGATG	XbaI-XhoI
TCM62	Fw_GTA TCTAGA ATGCTGAGGA ATTGTTTGAG GA Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCGGCTTTGTGCCGTTCAGGCTTTTTG	XbaI-BanHI
SPC2	Fw_GTATCTAGAATGAGTTCTGCTAAACCTATTAATG Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCTTCATTTTTTTTGGTGTCGAGGAC	XbaI-BamHI
CYB2	Fw_GTACCCGGG ATGCTAAAATACAAACCTTT ACT Rw_TATGAATTCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCTGCATCCTCAAATTCTGTTAAAGTAG	SmaI-EcoRI
SUR7	Fw_GTATCTAGAATGGTTAAGGTCTGGAATATAG Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCAACAGAGACATCGTCCGGGCGCTCG	XbaI-BamHI
AIM32	Fw_ TTCTCTAGAATGCTACGTATAACTGTCAAAAC Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCATTCATCGATATGCCACCTCTG	XbaI-XhoI
YAP6	Fw_TTCTCTAGAATGCAAAACCCTCCGTTGATTC Rw_TATCTCGAGCTCAGATCTTATCGTCGTCATCCTTGTAATC GCTGCCGCCGCCGCCCGTGGCACTATCCGAGTGTCTC	XbaI-XhoI
EXG2	Fw_TTCTCTAGAATGCCTTTGAAGTCGTTTTTTTTTTCAGC Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCAAGAGAAGCGCAAAGAGCGGCA	XbaI-XhoI
CRH1	Fw_TTCTCTAGAATGAAAGTGCTTGACCTACTAAC Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCTAGCAAGGCTAGTAAAGCTAC	XbaI-XhoI
YGR190C	Fw_TTCTCTAGAATGTCTTCCTGCTCTGTGTCTTC Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCAAACAACATATCATCAGCGTTA	XbaI-XhoI
HIP1	Fw_GTATCTAGAATGCCTAGAAACCCATTGAAAAAG Rw_TATGAATTCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCACACCAGAAATGTAAAAATCTTG	XbaI-EcoRI
RAD26	Fw_GTAACTAGTATGGAAGATAAAGAGCAGCAAG Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCTGAAGCATTGTTATTCCTAAATTC	SpeI-BamHI
HUL4	Fw_GTATCTAGAATGGTTTCTT TATTTGATAAGC Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCGCGGAACCCGTAACCTTCAGATTC	XbaI-BamHI
YJR039W	Fw_TTCTCTAGAATGCCAGCAGGGAGGATT Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCAATTTCAGATATTACAGTGTGA	XbaI-XhoI
FAT1	Fw_GTATCTAGAATGTCTCCCATACAGGTTGTTG Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCTAATTTAATTGTTTGTGCATCGATG	XbaI-BamHI
CST26	Fw_GTATCTAGAATGCTGCATCAAAAAATAGCTC Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCAAAAATAAAACAATAAAGTTTATAAC	XbaI-BanHI
QDR3	Fw_GTATCTAGAATGCAAGCCCAAGGTTCACAATC Rw_TATGGATCCTCACTTATCGTCGTCATCCTTGTAATCGCTG CCGCCGCCGCCGCCATCAATTTTGTCGTACAATTTTTG	XbaI-BamHI
YML054C-A	Fw_TTCTCTAGAATGATTCCCTTTCCTGCACAAC Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCCAGAGTCCAGGGCACAAGGGTC	XbaI-XhoI
YML053C	Fw_TTCTCTAGAATGCTCTCATACTATGAACACAA Rw_TATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCG CTGCCGCCGCCGCCATCTATATCCATGTAATCGTTAG	XbaI-XhoI

Results

Target Genes Identification by GWS Assay

To identify potential target genes able to interfere with the synergistic toxic effect mediated by the concomitant expression of ASYN and tau we used the "Yeast Genomic Tiling Collection Assay Ready DNA". The collection consists of over 1500 clones each containing a unique segment of the yeast *S. cerevisiae* genome in an *E. coli*-yeast shuttle vector provided as DNA pooled into one single tube. These plasmid clones represent a unique and virtually complete overlapping clone collection of the entire *S. cerevisiae* genome. This clone collection constitutes a "minimal functional pathway" through the yeast genome and represents ~97% of the length of the yeast genome at the physical level and ~95% of the genome at the functional level [28].

In solid media, the previously reported integrative yeast strain co-expressing ASYN WT and tau WT shows a strong synergistic toxic effect at 30°C, whereas total growth arrest is observed at 37°C [36]. Our goal now was to identify genes which expression in this model would lead to growth recovery, thus eliminating the observed toxicity. Transformation of the yeast strain co-expressing ASYN and tau with a vector harbouring the gene(s) able to eliminate this synergistic effect should lead to growth recovery. The tiling collection was transformed into our yeast model, followed by incubation in inducible solid media at 35°C. At this intermediate temperature, only cells with decreased cytotoxicity are able to survive. A total of three transformations resulted in 6 surviving colonies showing phenotype rescue by dot spot growth assay (Figure 1,A). In order to rule out a beneficial effect resulting from decreased expression of either ASYN or tau, the level of both proteins was evaluated by western blot (Figure 1,B). No significant differences in ASYN or tau proteins levels were observed for the 6 colonies that showed growth recovery upon transformation if compared with levels of the original yeast strain.

The plasmids carried by the recovering colonies were extracted and sequenced to identify the exact genomic fragments leading to phenotype rescue. According to the information provided by the tiling collection manufacturer, the 6 selected plasmids corresponded to 5 different vectors with a total number of 25 complete genes. Colonies 5 and 6 were carrying the same plasmid. Notably, the 25 genes identified included 4 genes with human ortholog, 4 dubious ORFs and 4 genes of unknown function (Table 2). The other 13 genes encompass many different molecular mechanisms ranging from cell wall assembly to gene expression.

Taking in consideration the gene function and the respective intracellular protein localization, 19 coding sequences (CDS) were selected for PCR amplification (Table 3). Preference was given to the genes having a human ortholog or linked to the typical neurodegenerative molecular mechanisms such as DNA damage, cell signalling and mitochondria activity.Selected CDS were added of a C-terminal FLAG epitope and individually sub-cloned into the p413 plasmid under the inducible Gal1 promoter. Each of the resulting vectors was then transformed into the yeast strain co-expressing ASYN and tau and growth ability tests were performed in order to identify the genes that, when expressed independently, were able to lead to phenotype recovery. Protein expression was then evaluated by immunoblotting using the anti-FLAG antibody (Figure 2).

Upon transformation, none of the constructs tested displayed beneficial effect for the cell viability (Figure 2,A) and protein expression was detectable by immunoblotting only in 8 samples (Figure 2,B). To further explore potential beneficial effect of the selected CDS that might be related to the protein expression level, we also used the constitutive GDP promoter to drive the expression of the selected CDS. The CDS of the constructs in which protein expression was detectable by immunoblotting were sub-cloned into the p413 GPD vector and transformed into the yeast strain co-expressing ASYN and tau. Again, by growth ability test no phenotype recovery was observed and protein expression was detectable in all the samples tested except in the strain transformed with the vector carrying the EXG2 CDS (data not shown).

Our first experimental approach to validate the single genes responsible for growth recovery was non successful and for many of the constructs tested protein expression was not detectable by anti-FLAG immunoblotting. However, these preliminary results suggest that to validate our data we might need to use other kind of constructs more similar to the collection vector in order to have an appropriate and functional protein expression.

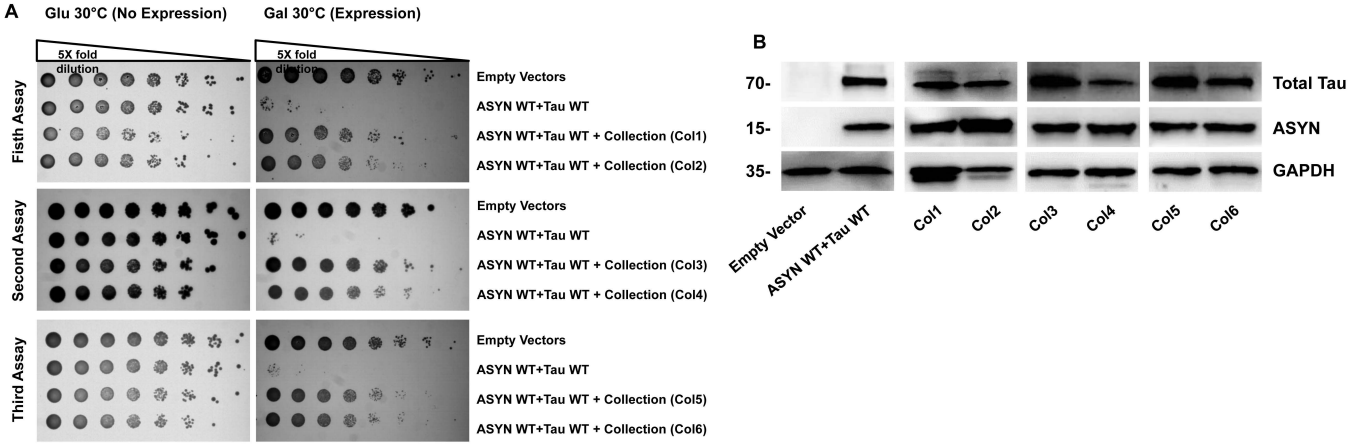

Figure 1: Transformants colonies viability assay. A) Six surviving colonies showing phenotype rescue by dot spots were identified upon transformation of the strain co-expressing ASYN WT and tau WT with the tiling collection. **B)** ASYN and tau protein levels remain similar between the reference strain (ASYN WT+Tau WT) alone or transformed with the tiling collection. The GAPDH level is increased in the Col1 as this strain is carrying the tiling collection vector containing the TDH3 gene, the yeast ortholog of the human GAPDH (Table 1). Results are representative of three independent experiments.

Table 2: Tiling collection vector identification. Upon sequencing 5 different vectors were identified with a total number of 25 complete genes, included 4 genes with human ortholog, 4 dubious ORFs and 4 genes of unknown function.

Collection ID	Trasformant Colony	Complete Genes Containded						
YGPM17f07	N°1	CRH1	YGR190C	HIP1	TDH3(GAPDH)			
YGPM29j05	N°2	RAD26	HUL4	YJR037W	YJR038C	YJR039W		
YGPM17j15	N°3	FIG1	FAT1	CST26	QDR3	TCM62		
YGPM7a09	N°4	SPC2	CYB2	YML054C-A	YML053C	SUR7	GAL80	AIM32
YGPM7p05	N°5 and 6	YAP6	SWM1	EXG2	YDR261W-A			

	With Human Ortholog
XXX	Unknown Function
	Dubious ORFs

Table 3: Genes individually tested. Summary of the individually cloned genes, intracellular protein localization, functions and promoters tested.

Gene	Protein Localization	Promoter Tested	Function
FIG1	Cell wall	Gal1-GPD	Ca2+ influx system, intracellular signaling and cell-cell fusion
EXG2	Cell wall	Gal1-GPD	Involved in cell wall beta-glucan assembly
CRH1	Cell wall	Gal1	Expression induced by cell wall stress
CST26	Cytoplasmic	Gal1	Incorporation of stearic acid into phosphatidylinositol and chromosome stability
YGR190C	Doubiuous ORF	Gal1	
SPC2	ER	Gal1-GPD	Subunit of the signal peptidase complex
FAT1	ER/Peroxisomes	Gal1	Very long chain fatty acyl-CoA synthetase and long chain fatty acid transporter
TCM62	Mitocondria	Gal1-GPD	Assembly of the mitochondrial succinate dehydrogenase complex
CYB2	Mitocondria	Gal1-GPD	Mitochondrial intermembrane space, required for lactate metabolism
YJR039W	Mitocondria	Gal1	Unknown
YAP6	Nuclear	Gal1-GPD	Transcription factor, regulation of genes involved in carbohydrate metabolism
RAD26	Nuclear	Gal1	Protein involved in repair of UV-induced DNA lesions
HUL4	Nuclear	Gal1	Ubiquitin-protein ligases
YML053C	Nuclear/Cytoplasmic	Gal1	Unknown
SUR7	Plasma memb	Gal1-GPD	Component of exosomes, associated with endocytosis
HIP1	Plasma memb	Gal1	High-affinity histidine permease, also involved in the transport of manganese ions
QDR3	Plasma memb	Gal1	Multidrug transporter of the major facilitator superfamily
AIM32	Unknown	Gal1-GPD	Unknown
YML054C-A	Unknown	Gal1	Unknown

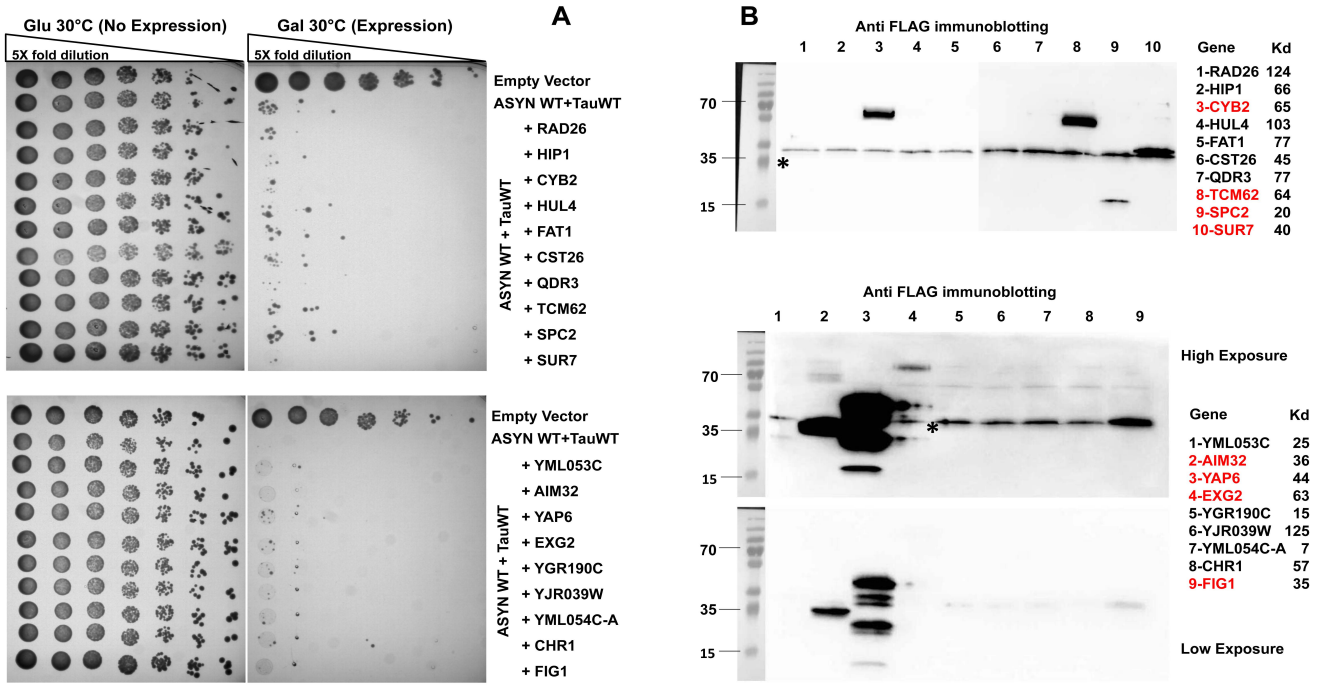

Figure 2: Screening viability assay. A) No phenotype rescue observed by dot spots upon transformation of the strain co-expressing ASYN WT and tau WT with the p413 Gal1 vector harbouring the 19 selected CDS. **B)** Protein expression was detectable by anti-FLAG immunoblotting only in 8 samples (in red), * corresponds to an unspecific band. Results are representative of three independent experiments.

Target Genes Identification by GWS Assay

Since our integrative yeast strain co-expressing ASYN and tau shows a strong and reproducible growth delay both in solid and liquid media [36], the model was also used to perform an HTS assay to identify natural extracts able to interfere with the observed synergistic toxic effect. The test collection was LUSOEXTRACT, a Portuguese natural heritage composed of 1206 organisms isolated from unique Portuguese terrestrial and aquatic ecosystems. From this collection, 1016 are microorganisms and 190 are marine invertebrates. All organisms that constitute the LUSOEXTRACT collection were obtained from collaborations between Bioalvo and the major Portuguese Universities and research centers, such as Faculdade de Ciências da Universidade de Lisboa (FCUL), Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT-UNL), Centro de Ciências do Mar da Universidade do Algarve (CCMAR), Universidade de Aveiro (UA), Faculdade de Ciências da Universidade do Porto (FCUP) and Instituto de Biologia Molecular e Celular (IBMC). A total of 3932 crude extracts were tested in this screening: 1094 methanol extracts, 942 dichloromethane extracts, 916 hexane extracts, 240 water extracts and 740 ethyl acetate extracts.

The integrative yeast strain co-expressing ASYN WT and tau WT (target) was used for the screening of the 3932 extracts. The target strain was grown in 96-well plates with 200 µl of liquid media at 30°C and, as expected, displayed a marked growth delay in comparison with the control strain carrying empty the vectors (control) (Figure 3). The goal of the screening was to identify natural extracts able to restore the growth of the target strain to levels closer to the ones of the control strain. The threshold OD600 value defined for classifying extracts as hits was: MAX + STDEV. MAX represents the maximum OD600 value measured of the target strain and STDEV is the standard deviation of all the OD600 values of the same strain. The time point chosen for hits picking was 60 hours, at this time the probability to pick false positive extracts is very poor as the control strain is already in stationary phase, the target strain is growing slowly and the threshold value is stable. Extracts resulting in OD600 value at 60 hours higher than this threshold were considered as hits (Figure 3). The Z factor calculated in the validation assay had a value of 0,79, which reflects the robustness of the platform and validates our system as a potent tool for HTS.

In the primary screening assay the LUSOEXTRACT crude extracts were added to the wells containing the target strain at a final concentration of 0,5 mg/ml and upon data analysis a total of 38 extracts out of the 3932 tested were classified as hits. In the secondary screening, the identified hits were re-tested in a confirmatory dose-response assay using four different final concentrations: 0,125 - 0,25 - 0,5 and 0,75 mg/ml. In this assay the control strain was used to test the hits at the higher concentration of 0,75 mg/ml in order in order to identify (and subsequently exclude) eventual false positives able to enhance the growth of yeast strains. Results obtained were analysed according to the same parameters used in the primary screening and 17 out of the 38 hits identified in the primary screening were re-confirmed. However, 4 hits also increased the growth of the control strain, thus they were excluded from the final readout which consists in 13 confirmed hits. To classify the hits, a ranking was established: very good (hits showing growth recovery for at least three concentrations), good (hits showing growth recovery for two concentrations) and weak (hits showing growth recovery for only one concentration) (Table 4). In order to rule out a beneficial effect on cell growth resulting from decreased expression of either ASYN or tau, the level of both proteins in yeast cells treated with the hit extracts was evaluated by western blot. No significant differences in ASYN or tau proteins levels were observed upon treatment with most of the extracts that showed growth recovery. However, a slight decrease in ASYN protein level was observed in the samples treated with extracts N°9 and N°13 (Figure 4).

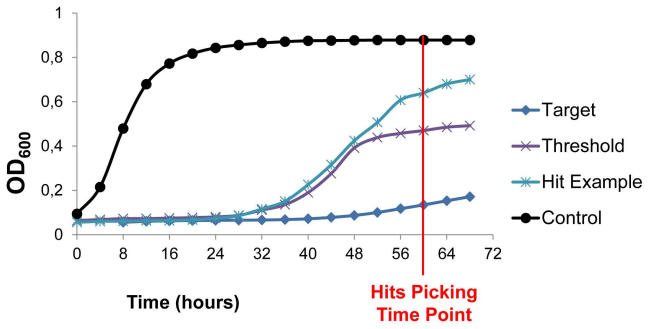

Figure 3: Example of a hit identification. The threshold chosen for hits determination was calculated as MAX + STDEV at 60 hours. Extracts resulting in OD600 values at 60 hours higher than this threshold were considered as hits.

Table 4: Extracts re-confirmed as hits. Summary of the hits identified the corresponding organism, specie and ranking.

LUSOEXTRACT Hits Confirmed	Organism	Specie	Ranking
Extract N° 1	Bacteria	*Rhodococcus sp.*	Very good
Extract N° 2	Anemones	*Calliactis parasitica*	Good
Extract N° 3	Bacteria	*Caulobacter leidyia*	Good
Extract N° 4	Filamentous fungi	*Mitosporic Ascomycota*	Good
Extract N° 5	Bacteria	*Rhodopirellula baltica*	Weak
Extract N° 6	Anemones	*Calliactis parasitica*	Weak
Extract N° 7	Filamentous fungi	*Fomitopsis spraguei*	Weak
Extract N° 8	Filamentous fungi	*Cladosporium*	Weak
Extract N° 9	Yeast	*Pichia pini*	Weak
Extract N° 10	Filamentous fungi	*Cladosporium*	Weak
Extract N° 11	Anemones	*Actinothoe spyrodeta*	Weak
Extract N° 12	Filamentous fungi	*Trametes pubescens*	Weak
Extract N° 13	Bacteria	*Rhodococcus sp.*	Weak

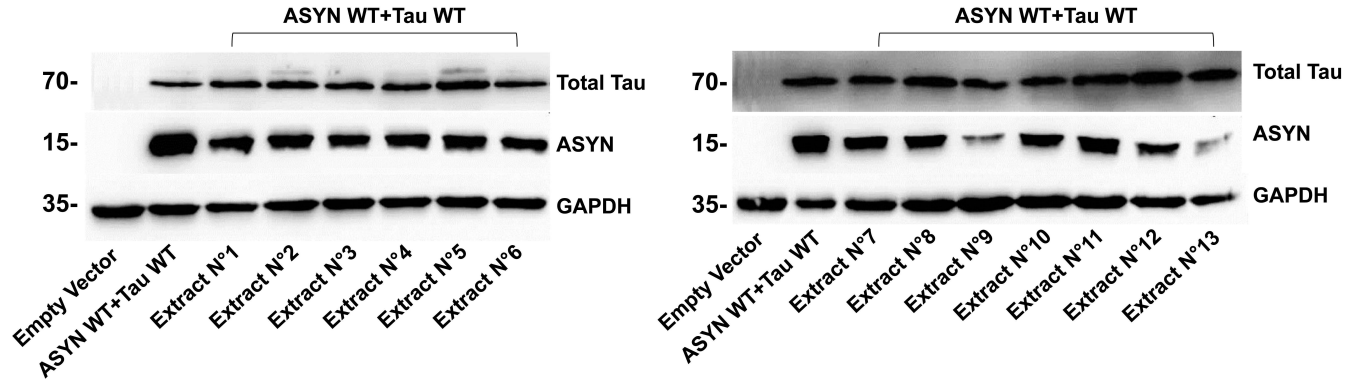

Figure 4: Protein expression analysis of the target strain treated with the hit extracts. ASYN and tau protein level remains constant in the samples threated with the extracts identified as hits at 0.5 mg/ml. Exception was observed upon treatment with the extracts N°9 and N°13 that showed a slight decrease in the ASYN protein level.

Interestingly, two of the confirmed hits correspond to extracts of Calliactis parasitica that produces neurotoxins such as Cpl and Calitoxin (CLX) [41,42]. Other relevant specie, Rhodopirelulla baltica, is able to degrade sulphated polymeric carbon in the marine environment [43]. Thus, our HTS resulted in 11 confirmed natural extracts hits coming from the unique Portuguese LUSOEXCTRAC collection, that have the potential to be further explored in other ASYN-tau disease related models

Discussion

Synucleinopathies and tauopathies regroup a wide number of neurodegenerative disorders such as AD and PD [1-3]. Synucleinopathies are characterized by the presence of Lewy bodies mainly composed by the presynaptic ASYN protein [10] whereas the pathological hallmark of tauopathies consists in the presence of neurofibrillary tangles positive for hyperphosphorylated tau protein [4-6]. Interactions between ASYN and tau have been shown to occur in several neurodegenerative disease models, suggesting that a synergistic neurotoxic effect might exist [11-16]. However, the mechanisms leading to cell death related to ASYN and tau interactions are still elusive. Yeast cells have been proven to be a powerful model to get insight into neurodegenerative disorders [30-32] and are largely used in genome-wide screening and high-throughput screening assay [24-29]. In our previous study we developed and characterized a stable yeast model in which the co-expression of the human ASYN and tau protein is synergistically cytotoxic [36]. Here, we used this model to perform a GWS and HTS assays in order to identify both target genes and natural extracts able to interfere with the cytotoxic effect observed.

The GWS assay was performed in solid media using the "Yeast Genomic Tiling Collection Assay Ready DNA" consisting of about 1500 clones, each containing a unique segment of the yeast *S. cerevisiae* genome in an *E. coli*-yeast shuttle vector provided as DNA pooled into one single tube [28]. Upon transformation of the yeast strain co-expressing ASYN and tau with the collection pooled clones, we were able to identify 6 surviving colonies showing phenotype recovery. The phenotype rescue was an effect completely mediated by the overexpression of *S. cerevisiae* genomic fragments and thus linked to the genes contained into, since the protein expression levels of ASYN and tau remained comparable between the yeast strains with or without collection vectors. To identify the genomic fragment contained in each surviving colony we extracted and sequenced the respective vectors. Data analysis revealed 5 different genomic fragments with a total number of 25 complete genes. To further validate our results discovering the genes responsible for growth recovery we decided to test individually each single gene and considering the gene function and cellular localization we selected 19 primary target CDS. We started the validation by cloning the selected FLAG-tagged CDS in a new vector under the inducible Gal1 or constitutive GPD promoter. However upon transformation none of the constructs tested showed growth recovery and in most of the samples protein expression was not detectable by anti-FLAG immunoblotting. These preliminary results suggest that to have a functional protein expression, comparable with the collection vector, and thus observe growth recovery, we might need to use other constructs

harbouring the endogenous promoter in combination with the respective complete ORF. It is also plausible that the phenotype rescue could be mediated not only by one single gene but it might be the resultant of cooperation between two or more genes carried in the same collection vector. However, the identification of genomic fragments that, when overexpressed, are able to strongly increase the cells lifespan, deserves further attention to uncover new genes and pathways that might be targeted for the treatment of neurodegenerative disorders.

The high stability of our model in combination with a reproducible strong growth delay also in liquid media [36], allowed us to perform a HTS assay using our in house LUSOEXCTRACT collection. The aim of the screening was to identify natural extracts able to enhance the cell growth of the yeast strain co-expressing the ASYN and tau protein. The screening was performed using an automated workstation and divided in two steps. In the first step the natural extracts were tested in a single dose and upon data analysis a total number of 38 extracts out of the 3932 tested were classified as hits. In the second step the 38 hits were re-tested in a confirmatory dose-response assay and data analysis resulted in 13 confirmed hits. A further validation assay was than performed to evaluate if the growth recovery resulted from decreased ASYN or tau protein expression levels and a slight decrease in ASYN protein level was observed in the samples treated with 2 out of the 13 confirmed hits. The final readout of our HTS assay resulted in 11 natural extracts able to alleviate the cytotoxic effect mediated by the concomitant expression of the human ASYN and tau proteins.

In summary, the high phenotype reproducibility makes our yeast model a powerful tool to perform various preliminary screening assays. In this study, the model versatility allowed us to identify a group of 25 potential target genes and 11 natural extracts, coming from organisms isolated from unique Portuguese terrestrial and aquatic ecosystems, which might be relevant for future therapeutic intervention strategies.

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Author Contributions

Conceived and designed the experiments: GC AM HV PC. Performed the experiments: GC MA. Analyzed the data: GC MA PC HV. Wrote the report: GC PC.

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Chapter III. General discussion, Future Perspectives and Conclusions

1. Discussion

In the last century the number of people affected by neurodegenerative disorders causing dementia in developed and developing countries is increasing year after year, mainly as consequence of increased life expectancy [1-3]. The term synucleinopathies regroups different neurodegenerative disorders characterized by the presence of insoluble fibrillary aggregates of ASYN protein, called Lewy bodies. This group of disorders includes Parkinson's disease, many cases of Alzheimer's disease (called Lewy bodies variant of Alzheimer's disease), Multiple System Atrophy, dementia with neurofibrillary tangles, neurodegeneration with brain iron accumulation type I, pure autonomic failure and a subtype of essential tremor [194,195]. Likewise, the term tauopathies represent another group of neurodegenerative disorders also characterized by the presence of neurofibrillary tangles labelling positive for tau protein [62]. Pathologies such as Alzheimer's disease, frontotemporal lobar degeneration, progressive supranuclear palsy and corticobasal degeneration are all examples of tauopathies [62]. Taken together, synucleinopathies and tauopathies account for about 70% of diseases causing dementia [1-3]. The indication of a potential synergistic neurotoxic effect between synucleinopathies and tauopathies rised up when in patients' brains ASYN was found within neurofibrillary tangles and tau within Lewy bodies [473-478]. Further investigations demonstrated a direct binding between ASYN and tau and the ability to promote each other's fibrillization [250,324,479-481]. Most importantly a synergistic cytotoxic effect upon co-expression of ASYN and tau has been reported both in mammalian and yeast models [347,482].

Given the devastating nature of neurodegenerative disorders and the lack of causal therapies, there is great need for finding effective treatments. Uncovering novel targets in the continuous search for new therapeutic treatments will enable us to move forward towards the development of more effective therapies against

the devastating disorders affecting the nervous system. In the work described in this thesis, we used yeast as a model system to reproduce and characterize the synergistic cytotoxic effect mediated by the co-expression of the human ASYN and tau proteins. The model was then used as a tool for the discovery of genes and compounds that mediate the toxic interaction between ASYN and tau opening up new targets for therapeutic intervention.

We started by promoting the expression of ASYN and tau in yeast from a bidirectional episomal vector. Our results showed that the presence of ASYN affected the solubility of tau increasing the fraction of insoluble/aggregated protein. Since tau phosphorylation is known to lead to the formation of insoluble tau aggregates and the pathological phospho-epitope S396/404 is a typical GSK3B substrate, already shown to be a target for tau phosphorylation in yeast, we analysed the tau phosphorylation at this specific epitope [470,503-507]. We showed that the presence of ASYN leads to increased tau phosphorylation at S396/404 via Rim11, the yeast ortholog of GSK3B. However, despite the enhanced phosphorylation and aggregation of tau promoted by co-expression of ASYN, no evident synergistic toxic effect was detected in this yeast strain. Looking for a synergistic cytotoxicity, we next evaluated the co-expression of ASYN and tau making use of a different experimental approach, based on the stable integration of one copy of each human transgene in the yeast genome. In this strain was achieved a strong synergistic toxic effect in yeast growth in association with the presence of insoluble/aggregated tau and increased phosphorylation of tau at S396/404 via Rim11 as a consequence of stimulation by ASYN. Importantly, the synergistic cytotoxicity is abrogated upon *RIM11* removal, thus validating our double transgenic model. Our results suggest that the synergistic cytotoxic effect might be the resultant of enhanced ASYN mediated tau phosphorylation at S396/404 that might give rise to potential cytotoxic oligomeric tau species. This is the first evidence that ASYN is able to induce tau phosphorylation and aggregation in yeast, proving that yeast recapitulates the reported mechanism through which ASYN stimulates GSK3B leading to phosphorylation of tau at pathogenic epitopes [484,485].

The yeast models presented here are powerful tools for rapid screening assays. In particular the integrative double transgenic yeast strain showing a strong synergistic toxic effect both in solid and liquid media was used to perform GWS and HTS assays to identify genes and compounds able to modulate the cytotoxic interactions between ASYN and tau proteins.

The GWS assay was performed using the "Yeast Genomic Tiling Collection Assay Ready DNA" consisting of about 1500 clones, each containing a unique segment of the yeast *S. cerevisiae* genome in an *E. coli*-yeast shuttle vector provided as DNA pooled into one single tube [495]. The screening resulted in the selection of 5 different *S. cerevisiae* genomic fragments that, when over-expressed, are able to increase the cells lifespan. Within these fragments a total number of 25 different complete genes have been identified. We next tried to discover the genes that, when expressed independently, were responsible for growth recovery. However, our first experimental approach was not successful as none of the single gene tested showed growth recovery and in most of the samples protein expression was not detectable. In this step, it is plausible that we might use other kind of constructs more similar to the collection vector in order to have an appropriate functional protein expression and thus growth recovery. It is also plausible that the phenotype rescue could be mediated not only by one single gene but it might be the resultant of cooperation between two or more genes carried in the same collection vector. The high stability of our model in combination with a reproducible strong growth delay also in liquid media, allowed us to perform a HTS assay using our in house LUSOEXCTRACT collection. This collection is a Portuguese natural heritage consisting in 3932 natural extracts coming from 1206 organisms isolated in the unique Portuguese terrestrial and aquatic ecosystems. The screening was divided in two steps. In the first step the natural extracts were tested in a single dose and upon data analysis a total number of 38 extracts were classified as hits. In the second step the hits were re-tested in a confirmatory dose-response assay and data analysis resulted in 13 confirmed hits. A further validation assay was than performed to evaluate if the growth recovery resulted from decreased ASYN or

tau protein expression levels. The final readout resulted in 11 natural extracts able to alleviate the cytotoxic effect mediated by the concomitant expression of the human ASYN and tau proteins.

In summary, the power of our yeast model allowed us to perform both GWS and HTS assays. The identification of genomic fragments that, when overexpressed, are able to strongly enhance the cells resistance to the ASYN and tau mediated cytotoxicity deserves further attention to uncover new genes and pathways that might be targeted for the treatment of neurodegenerative disorders. On the other hand, the 11 natural extracts confirmed as hits have the potential to be further explored in other ASYN-tau disease related models and developed to be used in the pharmacological treatments of patients affected by neurodegeneration.

2. Conclusions

Neurodegenerative brain diseases constitute a major health problem in Europe, and their impact on public health and society is increasing with the aging of the population. Various cellular and animal models of synucleinopathies and tauopathies have been created, showing neuronal and neurological dysfunction or emulating the physiopathology of neurodegenerative disorders. Despite these important insights, significant gaps in our understanding on ASYN and tau interactions and their relationship to the diseases progression still exist. Currently, there are no causal treatments available for PD, AD and other synucleinopathies or tauopathies. Therefore, novel therapeutic strategies need to be developed for these disease states.

Budding yeast have been successfully used to perform studies on neurodegenerative disorders that have resulted in the identification of several promising therapeutic drugs and targets. In this study we developed a yeast based screening platform to identify both genes and natural extracts able to interfere with synergistic cytotoxic effects mediated by the interaction of ASYN and tau. The identifications of modulators of ASYN and tau mediated synergistic toxicity might be not only relevant for academic groups working on neurodegeneration, but also for companies engaged in CNS drug discovery. Hopefully, our work will

impact patients, their families and care-takers significantly, however, would certainly need at least another 10 years to reach the patients.

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